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A potassium channel, the M-channel, as a therapeutic target.

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Compounds that stimulate or inhibit M-channels (ie, voltage-gated potassium channels formed by KCNQ2, KCNQ3 and KCNQ5) have been evaluated in clinical trials for epilepsy, stroke and Alzheimer's disease. The importance of M-channel function in reducing neuronal excitability is underscored by the finding that KCNQ2/3 mutations causing mild reduction of M-channel activity are linked to neonatal epilepsy. M-channel openers decrease the hyperexcitability responsible for epileptic seizures, neuropathic pain and migraine. Conversely, M-channel blockers may enhance cognitive functions. The M-channel has thus emerged as a promising target for treating epilepsy, stroke, migraine, pain, dementia, anxiety and bipolar disorder.

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◈ Potassium Channel Modulators

Numerous **potassium** channels have been reported. On the basis of the amino acid sequence of the pore-forming α subunit, **potassium** channels can be classified into two main superfamilies: the inward rectifier (K_{iR}) superfamily (including receptor-coupled, **ATP**-sensitive and voltage-dependent channels) and the *Shaker*-related superfamily (which includes Ca^{2+}-dependent channels).

ATP-Activated	Ca2+-Activated	Voltage-Gatede
Inward rectifiers	Other	

1. ATP-Activated

Compound	Pharmacological Action	References
Cromakalim BRL 34915 / *trans*-3,4-dihydro-3-hydroxy-2,2-dimethyl-4-(2-oxo-1-pyrrolidinyl)-2*H*-1-benzopyran-6-carbonitrile M.W. 286.33 Store at RT Soluble to 20 mM in DMSO [94470-67-4]	**Cat. No. 1377** Prototypical K_{ATP} **channel opener**. Relaxes rabbit isolated portal vein with an IC_{50} value of 21 nM. Potent, orally active and hypotensive in vivo.	**Escande** *et al* (1988) The **potassium channel opener** cromakalim (BRL 34915) activates **ATP**-dependent K^+ channels in isolated cardiac myocytes. Biochem.Biophys.Res.Comm. ***154*** 620. **Wilson** *et al* (1988) Comparative effects of K^+ **channel** blockade on the vasorelaxant activity of cromakalim, pinacidil and nicorandil. Eur.J.Pharmacol. ***152*** 331. **Longman** *et al* (1988) Cromakalim, a **potassium channel** activator: a comparison of its cardiovascular haemodynamic profile and tissue specificity with those of pinacidil and nicorandil. J.Cardiovasc.Pharmacol. ***12*** 535.
Diazoxide 7-Chloro-3-methyl-2H-1,2,4-benzothiadiazine 1,1-dioxide M.W. 230.67 Store at RT Soluble to 100 mM in DMSO	**Cat. No. 0964** Antihypertensive. Activates **ATP**-dependent K^+ channels; blocks desensitization of AMPA receptors.	Merck Index ***12*** 3051. **Trube** *et al* (1986) Opposite effects of tolbutamide and diazoxide on the **ATP**-dependent K^+ **channel** in mouse pancreatic β-cells. Pflugers Arch. ***407*** 493. **Yamada and Rothman** (1992) Diazoxide blocks

[364-98-7]		glutamate desensitization and prolongs excitatory postsynaptic currents in rat hippocampal neurons. J.Physiol. ***458*** 409.
Glibenclamide Glyburide / 5-Chloro-N-[2-[4-[[[(Cylcohexylamino)carbonyl] amino]sulphonyl]phenyl]ethyl]-2-methoxybenzamide M.W. 494.01 Store at RT Soluble to 5 mM in ethanol [10238-21-8]	**Cat. No. 0911** Blocks **ATP**-dependent K^+ channels in the pancreas, causing an increase in intracellular Ca^{2+} and insulin secretion.	**Brogden** *et al* (1979) Glibenclamide: a review. Drugs ***18*** 329. **Robertson** *et al* (1990) **Potassium channel** modulators: scientific applications and therapeutic promise. J.Med.Chem. ***33*** 1529.
Levcromakalim BRL 38227 / (3*S*,4*R*)-3,4-dihydro-3-hydroxy-2,2-dimethyl-4-(2-oxo-1-pyrrolidinyl)-2*H*-1-benzopyran-6-carbonitrile M.W. 286.33 Store at RT Soluble to 10 mM in DMSO [94535-50-9]	**Cat. No. 1378** Active enantiomer of the prototypical K_{ATP} **channel opener** cromakalim (catalogue number 1377). Hypotensive and airways relaxant. IC_{50} = 490 nM in guinea pig trachea.	**Buckle** *et al* (1991) Synthesis and smooth muscle relaxant activity of a new series of **potassium channel** activators: 3-amido-1,1-dimethylindan-2-ols. J.Med.Chem. ***34*** 919. **Noach** *et al* (1992) **Potassium channel** modulation in rat portal vein by **ATP** depletion: a comparison with the effects of levcromakalim (BRL 38227). Br.J.Pharmacol. ***107*** 945. **Taylor** *et al* (1992) The inhibitory effects of cromakalim and its active enantiomer BRL 38227 against various agonists in guinea pig and human airways. J.Pharmacol.Exp.Ther. ***261*** 429.
Minoxidil 6-(1-Piperidinyl)-2,4-pyrimidinediamine 3-oxide M.W. 209.25 Store at RT Soluble in ethanol [38304-91-5]	**Cat. No. 0583** Antihypertensive. Antialopecia agent. K^+ **channel** activator.	Merck Index ***12*** 6290. **Meisheri** *et al* (1993) Enzymatic and non-enzymatic sulfation mechanisms in the biological actions of minoxidil. Biochem.Pharmacol. ***45*** 271. **Tsoporis** *et al* (1993) Effects of the arterial vasodilator minoxidil on cardiovascular structure and sympathetic activity in spontaneously hypertensive rats. J.Hypertens. ***11*** 1337. **Lachgar** *et al* (1998) Minoxidil upregulates the expression of vascular endothelial growth factor in human hair dermal papilla cells. Br.J.Dermatol. ***138*** 407.
P1075 *N*-cyano-*N'*-(1,1-dimethylpropyl)-*N''*-3-pyridylguanidine M.W. 231.30 Store at RT Soluble to 100 mM in 1 eq. HCl and to 50 mM in ethanol Purity > 99% [60559-98-0]	**Cat. No. 1355** Potent K_{ATP} **channel opener** (EC_{50} for relaxation of rat aorta = 7.5 nM). Binds to SUR2A and SUR2B with high affinity (K_d values are 17 and 3 nM respectively).	**Higdon** *et al* (1997) Tissue and species variation in the vascular receptor binding of ^{3}H-P1075, a potent K_{ATP} **opener** vasodilator. J.Pharmacol.Exp.Ther. ***280*** 255. **Gross** *et al* (1999) Stoichiometry of **potassium channel opener** action. Mol.Pharmacol. ***56*** 1370. **Buckner** *et al* (2000) Pharmacological and molecular analysis of **ATP**-sensitive K^+ channels in the pig and human detrusor. Eur.J.Pharmacol. ***400*** 287. **Ashcroft and Gribble** (2000) New

		windows on the mechanism of action of KATP **channel** openers. TiPS ***21*** 439.
[^{3}H]-P1075 *N*-cyano-*N'*-(1,1-dimethyl-[2,3-^{3}H]-propyl)-*N''*-3-pyridylguanidine Unlabelled M.W. 231.3 Specific activity: 60-120 Ci/mmol(2.22-4.44 T Bq/mmol) Solvent: ethanol:water (1:1) Radiochemical Purity: > 97% Shipped in dry ice	**Cat. No R1355** Tritiated version of the selective K_{ATP} **channel opener** P1075 (Cat. No. 1355); binds with high affinity (K_d values are 3 and 6.1 nM in rat aorta and glomerulus respectively). Binding is temperature- and **ATP** concentration-sensitive but insensitive to membrane potential.	Bray and Quast (1992) A specific binding site for K^+ **channel** openers in rat aorta. J.Biol.Chem. 267 11689. Quast et al (1993) Binding of the K^+ **channel opener** [^{3}H]P1075 in rat isolated aorta: relationship to functional effects of openers and blockers. Mol.Pharmacol. 43 474. Metzger and Quast (1996) Binding of [^{3}H]P1075 , an **opener** of **ATP**-sensitive K^+ channels, to rat glomerular preparations. Naunyn-Schmied.Arch.Pharmacol. 354 452. Higdon et al (1997) Tissue and species variation in the vascular receptor binding of ^{3}H-P1075, a potent K_{ATP} **opener** vasodilator. J.Pharmacol.Exp.Ther. 280 255.
Pinacidil *N*-Cyano-*N*?4-pyridinyl-*N*╡-(1,2,2-trimethylpropyl)-guanidine M.W. 245.33 Store at RT Soluble to 100 mM in ethanol and to 100 mM in DMSO [60560-33-0]	**Cat. No. 1503** K^+ **channel opener** and vasodilator. Activates K_{ATP} channels (IC_{50} for relaxation of coronary arteries = 1.26 µM). Clinically used antihypertensive agent.	**Petersen** (1978) Synthesis and hypotensive activity of *N*-alkyl-*N*╡-cyano-*N*?pyridylguanidines. J.Med.Chem. ***21*** 773. **Kimura** *et al* (1988) Effects of an antihypertensive vasodilator, pinacidil, on regional blood flow in concious spontaneously hypertensive rats. J.Pharmacobiodyn. ***11*** 430. **Gollasch** *et al* (1995) Pinacidil relaxes porcine and human coronary arteries by activating **ATP**-dependent **potassium** channels in smooth muscle cells. J.Pharmacol.Exp.Ther. ***275*** 681.
ZM 226600 N-(4-Phenylsulphonylphenyl)-3,3,3-trifluoro-2-hydroxy-2-methylpropanamide M.W. 373.35 Store at RT Soluble in ethanol	**Cat. No. 0882** Potent K_{ATP} **channel opener** (EC_{50} = 0.5 µM), devoid of anti-androgen properties.	**Grant** *et al* (1994) Anilide tertiary carbinols: a novel series of K^+ **channel** openers. TiPS ***15*** 402.

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2. Ca2+-Activated

Compound	Pharmacological Action	References
Apamin M.W. 2027.34 $C_{79}H_{131}N_{131}O_{24}S_4$ Desiccate at -20°C Solubility: see peptides [24345-16-2]	**Cat. No 1652** Prototypical potent and highly selective inhibitor of the small-conductance Ca^{2+}-activated K^+-**channel** (SK_{Ca}). Blocks	**Habermann** (1984) Apamin. Pharmac.Ther. ***25*** 255. **Strong** (1990) **Potassium channel** toxins. Pharmac.Ther. ***46*** 137. **van der Staay** *et al* (1999) Behavioral effects of apamin, a selective inhibitor of the SK_{Ca}-**channel**, in

	slow after-hyperpolarisation in vitro and is brain penetrant and convulsive in vivo.	mice and rats. Neurosci.Biobehav.Rev. ***23*** 1087.
Charybdotoxin M.W. 4295.90 Desiccate at -20°C Solubility: see Peptides [95751-30-7]	**Cat. No. 1087** Specific inhibitor of the high conductance Ca^{2+}-activated K^+ **channel**.	**Miller** *et al* (1985) Charybdotoxin, a protein inhibitor of single Ca^{2+} activated K^+ channels from mammalian skeletal muscle. Nature ***313*** 316. **Gimenez-Gallego** *et al* (1988) Purification, sequence, and model structure of charybdotoxin, a potent selective inhibitor of calcium activated **potassium** channels. Proc.Natl.Acad.Sci.USA ***85*** 3329. **Asano** *et al* (1993) Charybdotoxin-sensitive K^+ channels regulate the myogenic tone in the resting state of arteries from spontaneously hypertensive rats. Br.J.Pharmacol. ***108*** 214.
DCEBIO 5,6-Dichloro-1-ethyl-1,3-dihydro-2*H*-benzimidazol-2-one M.W. 231.08 Store at RT Soluble in DMSO [60563-36-2]	**Cat. No. 1422** Stimulates Cl^- secretion via activation of hIK1 K^+ channels and the activation of an apical Cl^- conductance. More potent than its analogue 1-EBIO.	**Singh** *et al* (2001) Benzimidazolone activators of chloride secretion: potential therapeutics for cystic fibrosis and chronic obstructive pulmonary disease. J.Pharmacol.Exp.Ther. ***296*** 600.
Dequalinium dichloride 1,1´-(1,10-Decanediyl)bis (4-amino-2-methylquinolinium) chloride M.W. 527.58 Store at RT Slightly soluble in water [522-51-0]	**Cat. No. 0674** A potent and selective non-peptide blocker of the apamin-sensitive small conductance Ca^{2+}-activated K^+ **channel**.	**Dunn** (1994) Dequalinium, a selective blocker of the slow afterhyperpolarization in rat sympathetic neurones in culture. Eur.J Pharmacol. ***252*** 189.
1-EBIO 1-Ethyl-2-benzimidazolinone M.W. 162.19 Store at RT Soluble to 100 mM in DMSO [10045-45-1]	**Cat. No. 1041** The first activator of epithelial K_{ca} channels to be identified, across T84 monolayers it stimulates a large and sustained trans-epithelial chloride secretory response.	**Devor** *et al* (1996) Modulation of Cl- secretion by benzimidazolones. I. Direct activation of a Ca^{2+}-dependent K^+ **channel**. Am.J.Physiol. ***271*** L775. **Devor** *et al* (1996) Modulation of Cl- secretion by benzimidazolones. II. Coordinate regulation of apical G_{Cl} and basolateral G_K . Am.J.Physiol. ***271*** L785. **Ayotunde and Adeagbo** (1999) 1-Ethyl-2-benzimidazolinone stimulates endothelial K_{Ca} channels and nitric oxide formation in rat mesenteric vessels. Eur.J.Pharmacol. ***379*** 151.
Iberiotoxin M.W. 4230 Desiccate at -20°C Solubility: see Peptides [129203-60-7]	**Cat. No. 1086** Selective blocker of the high conductance Ca^{2+}-activated K^+ **channel**.	**Galvez** *et al* (1990) Purification and characterization of a unique, potent, peptidyl probe for the high conductance calcium-activated **potassium channel** from venom of the scorpion *Buthus tamulus*.

		J.Biol.Chem. ***265*** 1108. **Suarez-Kurtz** *et al* (1991) Effects of charybdotoxin and iberiotoxin on the spontaneous motility and tonus of different guinea-pig smooth muscle tissues. J.Pharmacol.Exp.Ther. ***259*** 439.
Paxilline M.W. 435.56 $C_{27}H_{33}NO_4$ Desiccate at -20°C [57186-25-1]	**Cat. No 2006** Potent blocker of high-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels. Binds to the α-subunit of BK_{Ca} (K_i = 1.9 nM for block of currents in α-subunit-expressing oocytes) and enhances binding of charybdotoxin to BK_{Ca} channels in vascular smooth muscle. Also inhibits sarco/endoplasmic reticulum Ca^{2+}-ATPase (IC_{50} = 5-50 μM).	**Knaus** *et al* (1994) Tremorgenic indole alkaloids potently inhibit smooth muscle high-conductance calcium-activated **potassium** channels. Biochemistry ***33*** 5819. **Sanchez and McManus** (1996) Paxilline inhibition of the alpha-subunit of the high-conductance calcium-activated **potassium channel**. Neuropharmacology ***35*** 963. **Bilmen** *et al* (2002) The mechanism of inhibition of the sarco/endoplasmic reticulum Ca^{2+} ATPase by paxilline. Arch.Biochem.Biophys. ***406*** 55.
UCL 1684 6,12,19,20,25,26-Hexahydro-5,27:13,18:21,24-trietheno-11,7-metheno-7*H*-dibenzo [b,n] [1,5,12,16]tetraazacyclotricosine-5,13-diium ditrifluoroacetate M.W. 720.67 $C_{38}H_{30}N_4F_6O_4$ Desiccate at Room temperature Soluble to 10 mM in DMSO [199934-16-2]	**Cat. No 1310** Highly potent, non-peptidic blocker of the apamin-sensitive Ca^{2+}-activated K^+ **channel** (SK_{Ca}) (IC_{50} = 3 nM in rat sympathetic neurons). Blocks hSK1 and rSK2 channels expressed in HEK 293 cells with IC_{50} values of 762 and 364 pM respectively.	**Campos Rosa** *et al* (2000) Synthesis, molecular modeling, and pharmacological testing of bis-quinolinium cyclophanes: potent, non-peptidic blockers of the apamin-sensitive Ca^{2+}-activated K^+ **channel**. J.Med.Chem. ***43*** 420. **Malik-Hall** *et al* (2000) Compounds that block intermediate-conductance (IK_{Ca}) and small-conductance (SK_{Ca}) calcium-activated **potassium** channels. Br.J.Pharmacol. ***129*** 1431. **Strobaek** *et al* (2000) Pharmacological characterization of small-conductance Ca^{2+}-activated K^+ channels stably expressed in HEK 293 cells. Br.J.Pharmacol. ***129*** 991.

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3. Voltage-Gated

Compound	Pharmacological Action	References
AM 92016 hydrochloride 1-(4-Methanesulphonamidophenoxy)-3-(N-methyl-3,4-dichlorophenylethylamino)-2-propanol M.W. 483.84 Store at RT Soluble to 100 mM in water and to 100 mM in ethanol [178894-81-0]	**Cat. No. 0876** A specific blocker of the time dependent delayed rectifier **potassium** current, devoid of any β-adrenoceptor blocking activity.	**Conners** *et al* (1992) Actions and mechanisms of action of novel analogues of sotalol on guinea-pig and rabbit ventricular cells. Br.J.Pharmacol. ***106*** 958. **White** *et al* (1993) The positive inotropic effect of compound II, a novel analogue of sotalol, on guinea-pig papillary muscles and single ventricular myocytes.

		Br.J.Pharmacol. ***110*** 95. **Hagerty** *et al* (1996) The *in vivo* cardiovascular effects of a putative class III anti-arrhythmic drug, AM 92016. J.Pharm.Pharmacol. ***48*** 417. **Lei and Brown** (1998) Inhibition by compound II, a sotalol analogue, of delayed rectifier current (i_k) in rabbit sino-atrial node cells. Naunyn-Schmied.Arch.Pharmacol. ***357*** 260.
Chromanol 293B *trans*-*N*-[6-Cyano-3,4-dihydro-3-hydroxy-2,2-dimethyl-2*H*-1-benzopyran-4-yl]-*N*-methyl-ethanesulfonamide M.W. 324.39 Store at RT Soluble in DMSO [163163-23-3]	**Cat. No. 1412** Blocker of the slow delayed rectifier K^+ current (I_{Ks}) (IC_{50} = 1-10 μM). Also blocks the CFTR chloride current (I_{CFTR}) (IC_{50} = 19 μM).	**Fujisawa** *et al* (2000) Time-dependent block of the slowly activating delayed rectifier K(+) current by chromanol 293B in guinea-pig ventricular cells. Br.J.Pharmacol. ***129*** 1007. **Backmann** *et al* (2001) Chromanol 293B, a blocker of the slow delayed rectifier K^+ current (I_{KS}), inhibits the CFTR Cl- current. Naunyn-Schmied.Arch.Pharmacol. ***363*** 590. **Sun** *et al* (2001) Chromanol 293B inhibits slowly activating delayed rectifier and transient outward currents in canine left ventricular myocytes. J.Cardiovasc.Electrophysiol. ***12*** 472.
(-)-[3*R*,4*S*]-Chromanol 293B *N*-[(3*R*,4*S*)-6-Cyano-3,4-dihydro-3-hydroxy-2,2-dimethyl-2*H*-1-benzopyran-4-yl]-*N*-methyl M.W. 324.39 Store at RT Soluble to 1 mM in ethanol and 1 mM in DMSO [163163-24-4]	**Cat. No. 1475** Enantiomer that selectively inhibits the slow component of delayed rectifier K^+ current (I_{Ks}). Block is use-dependent and 7-fold more potent than the (+)-(3*S*,4*R*) enantiomer (IC_{50} values are 1.36 and 9.6 μM respectively). Has negligible inhibitory action at hERG channels (IC_{50} > 30 μM).	**Yang** *et al* (2000) Stereoselective interactions of the enantiomers of chromanol 293B with human voltage-gated **potassium** channels. J.Pharmacol.Exp.Ther. ***294*** 955. **Seebohm** *et al* (2001) A kinetic study on the stereospecific inhibition of KCNQ1 and I_{Ks} by the chromanol 293B. Br.J.Pharmacol. ***134*** 1647. **Lerche** *et al* (2001) Molecular impact of MinK on the enantiospecific block of *I*Ks by chromanols. Br.J.Pharmacol. ***131*** 1503.
CP 339818 hydrochloride *N*-[1-(Phenylmethyl)-4(1*H*)-quinolinylidene]-1 pentanamine hydrochloride M.W. 340.89 Store at RT Soluble to 20 mM in water [185855-91-8]	**Cat. No. 1399** Potent, non-peptide Kv1.3 **channel** antagonist that preferentially binds to the C-type inactivated state of the **channel** (IC_{50} ~ 200 nM). Inhibits Kv1.4 with an IC_{50} of ~ 300 nM. Selective over Kv1.1, Kv1.2, Kv1.5, Kv1.6, Kv3.1-4 and Kv4.2.	**Nguyen** *et al* (1996) Novel nonpeptide agents potently block the C-type inactivated conformation of Kv1.3 and suppress T cell activation. Mol.Pharmacol. ***50*** 1672. **Jager** *et al* (1998) Regulation of mammalian *Shaker*-related K^+ channels: evidence for non-conducting closed and non-conducting inactivated. J.Physiol. ***506*** 291.
Linopirdine dihydrochloride 1,3-Dihydro-1-phenyl-3,3-*bis*(4-pyridinylmethyl)-2*H*-indol-2-one	**Cat. No 1999** Blocker of **KCNQ** voltage-	**Schnee and Brown** (1998) Selectivity of linopirdine (DuP 966), a neurotransmitter release

dihydrochloride DuP 996 M.W. 464.39 $C_{26}H_{21}N_3O.2HCl$ Desiccate at Room temperature Soluble to 100 mM in water Purity: > 99% [105431-72-9]	gated **potassium** channels; blocks KCNQ2+3/M-currents (IC_{50} = 4-7 μM) and KCNQ1 homomeric channels (IC_{50} = 8.9 μM). Augments hippocampal ACh release and is a cognitive enhancer following oral administration in vivo.	enhancer, in blocking voltage-dependent and calcium-activated **potassium** currents in hippocampal neurons. J.Pharmacol.Exp.Ther. ***286*** 709. **Wang** *et al* (1998) KCNQ2 and KCNQ3 **potassium channel** subunits: molecular correlates of the M-**channel**. Science ***282*** 1890. **Zaczek** *et al* (1998) Two new potent neurotransmitter release enhancers, 10,10-bis(4-pyridinylmethyl)-9(10H)-anthracenone and 10,10-bis(2-fluoro-4-pyridinylmethyl)-9(10H)-anthracenone: comparison to linopirdine. J.Pharmacol.Exp.Ther. ***285*** 724.
XE 991 dihydrochloride 10,10-*bis*(4-Pyridinylmethyl)-9(10*H*)-anthracenone dihydrochloride M.W. 449.37 $C_{26}H_{20}N_2O.2HCl$ Desiccate at Room temperature Soluble to 100 mM in water Purity: > 99% [122955-42-4]	**Cat. No 2000** Potent and selective blocker of **KCNQ** voltage-gated **potassium** channels. Blocks KCNQ2+3/M-currents (IC_{50} = 0.6-0.98 μM) and KCNQ1 homomeric channels (IC_{50} = 0.75 μM) but is less potent against KCNQ1/minK channels (IC_{50} = 11.1 μM). Augments hippocampal ACh release and is a cognitive enhancer following oral administration in vivo.	**Wang** *et al* (1998) KCNQ2 and KCNQ3 **potassium channel** subunits: molecular correlates of the M-**channel**. Science ***282*** 1890. **Zaczek** *et al* (1998) Two new potent neurotransmitter release enhancers, 10,10-bis(4-pyridinylmethyl)-9(10H)-anthracenone and 10,10-bis(2-fluoro-4-pyridinylmethyl)-9(10H)-anthracenone: comparison to linopirdine. J.Pharmacol.Exp.Ther. ***285*** 724. **Wang** *et al* (2000) Molecular basis for differential sensitivity of **KCNQ** and I_{Ks} channels to the cognitive enhancer XE991. Mol.Pharmacol. ***57*** 1218. **Passmore** *et al* (2003) **KCNQ**/M currents in sensory neurons: significance for pain therapy. J.Neurosci. ***23*** 7227.

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4. Inward rectifiers

Compound	Pharmacological Action	References
Tertiapin-Q M.W. 2452.00 Desiccate at -20°C Solubility: see Peptides	**Cat. No. 1316** A high affinity inhibitor for inward-rectifier K^+ channels, this compound is a stable derivative of the bee venom toxin tertiapin. Binds to ROMK1 and GIRK1/4 channels with high affinity (K_i values are 1.3 and 13.3 nM respectively) and is selective over IRK1 channels.	**Jin and Lu** (1999) Synthesis of a stable form of tertiapin: a high-affinity inhibitor for inward-rectifier K^+ channels. Biochemistry ***38*** 14286. **Jin** *et al* (1999) Mechanisms of inward-rectifier K^+ **channel** inhibition by tertiapin-Q. Biochemistry ***38*** 14294.

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5. Other

Compound	Pharmacological Action	References

4-Aminopyridine M.W. 94.12 Store at RT Soluble in DMSO [504-24-5]	**Cat. No. 0940** K^+-**channel** blocker.	**Bouchard and Fedida** (1995) Closed and open state binding of 4-aminopyridine to the cloned human **potassium channel** Kv1.5. J.Pharmacol.Exp.Ther. ***275*** 864. **Tseng** *et al* (1996) Reverse use dependence of Kv4.2 blockade by 4-aminopyridine. J.Pharmacol.Exp.Ther. ***279*** 865. **Tseng** (1999) Different state dependencies of 4-aminopyridine binding to rKv1.4 and rKv4.2: role of the cytoplasmic halves of the fifth and sixth transmembrane segments. J.Pharmacol.Exp.Ther. ***290*** 569.
SG 209 N-[2-(Acetoxy)ethyl]-3-pyridinecarboxamide M.W. 207.20 Desiccate at +4°C Soluble in water [83440-03-3]	**Cat. No. 0385** Analogue of nicorandil. A nitrate-free coronary vasodilator; activates K^+ channels.	**Ishibashi** *et al* (1991) 2-Nicotinamidoethyl acetate is a **potassium channel opener**: structure activity relationship among nicorandil derivatives. Naunyn-Schmied.Arch.Pharmacol. ***344*** 235.
YS-035 hydrochloride N,N-Bis-(3,4-dimethoxyphenylethyl)-N-methylamine M.W. 395.90 Desiccate at RT Soluble to 100 mM in water [33978-72-2]	**Cat. No. 0416** A Ca^{2+} blocker that strikingly prolongs cardiac action potentials by inhibiting pacemaker current and K^+ outward currents.	**Deana** *et al* (1984) Properties of a new calcium ion antagonist on cellular uptake and mitochondrial efflux of calcium ions. Biochem.J. ***218*** 899. **Berger** *et al* (1991) Inhibition of **potassium** outward currents and pacemaker current in sheep cardiac Purkinje fibres by the verapamil derivative YS-035. Naunyn-Schmied.Arch.Pharmacol. ***344*** 653.

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Potassium Channel Openers

Agents for the Treatment of Airway Hyperreactivity

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Summary

ATP-sensitive potassium (K_{ATP}) channels link the metabolic status of the cell to the plasma membrane potential and thus play a key role in regulating cellular excitability. K_{ATP} channel openers (KCOs) may impact positively on respiratory disease by suppressing bronchoconstriction, mucus hypersecretion, cough and airway hyperreactivity (AHR). A major recent development is the emergence of KCOs which can obviate experimental AHR at doses which are devoid of cardiovascular effects. This new generation of compounds with selectivity for the airways may constitute a new class of drugs for the treatment of asthma.

ATP-sensitive potassium (K_{ATP}) channels are found in a wide variety of tissues, including skeletal and smooth muscle cells, secretory cells (such as insulin-secreting pancreatic β-cells), cardiac myocytes and neurons [1–3]. Conceptually, the presence of K_{ATP} channels in bronchiolar smooth muscle [2] and airway sensory and autonomic neurons [4] raises the possibility of their involvement in the pathophysiology of respiratory disease through the modulation of direct and reflex-induced bronchoconstriction [5], mucus secretion [6] and cough [7]. In practice, it is the phenomenon of airway hyperreactivity (AHR), whose underlying mechanisms remain ill-defined [8, 9], which is emerging as the key target with clinical relevance to respiratory disease [10]. For this reason, the emphasis in this review will be placed on the role and therapeutic significance of K_{ATP} channels in the phenomenon of AHR.

Mechanism of Action

Potassium channel openers (KCOs) act by stimulating ion flux through a distinct class of potassium channels which are inhibited by intracellular ATP and activated by intracellular nucleoside diphosphates. Such K_{ATP} channels link the metabolic status of the cells to the plasma membrane potential and in this way play a key role in regulating cellular activity [1–3]. In most excitatory cells, K_{ATP} channels are closed under normal physiological conditions and open when the tissue is metabolically compromised (e.g. when the [ATP]:[ADP] ratio falls). This promotes K^+ efflux and the cell hyperpolarizes, thereby preventing voltage-operated Ca^{2+} channels (VOCs) from opening (fig. 1). K_{ATP} channels are composed of a pore-forming tetrameric complex of inward rectifying K^+ channel (K_{ir}) subunits (either $K_{ir}6.1$ or $K_{ir}6.2$), with each subunit being associated with a regulating protein of the sulphonylurea receptor type (SUR1, SUR2A or SUR2B) [11, 12] (table 1). SUR proteins are members of the ATP-binding-cassette transporter family and their nucleotide-binding domains are believed to render the K_{ATP} channels sensitive to [ATP]/[ADP]. SURs are also the target proteins for KCOs [14]. It is likely that the various combinations of SUR and $K_{ir}6$ subunits account for the clear differences between K_{ATP} channels in various tissues, with respect to their channel properties and sensitivity to ligands which both activate and inhibit their opening (table 1).

Range of Therapies

K_{ATP} channels are activated by a diverse group of compounds which include the anti-hypertensive agents, minoxidil sulphate, diazoxide and pinacidil, as well as a variety of benzopyran derivatives such as levcromakalim (or its racemate, cromakalim), SDZ PCO 400, bimakalim, JTV 506, YM 934, KC-399, BRL 55834, rilmakalim and SDZ 217-744 (fig. 2). Only the benzopyran derivatives have been profiled as therapies for asthma [15]. K_{ATP} channels are inhibited by sulphonylurea derivatives, such as glibenclamide and tolbutamide, and the high affinity of these agents for K_{ATP} channels in pancreatic β-cells is the

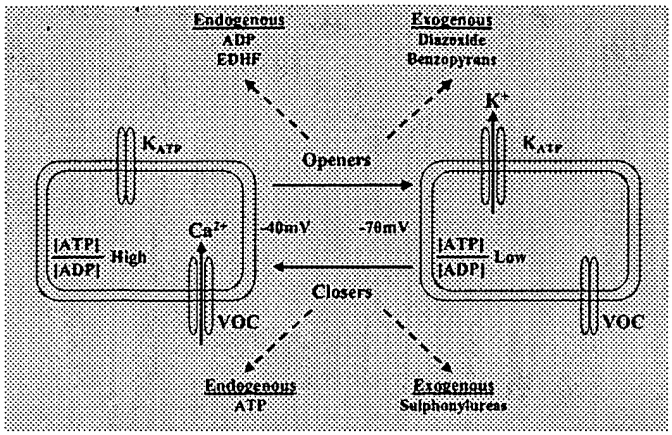

Fig. 1. Diagrammatic representation of the role of K_{ATP} channels in cell excitability. For further details see text.

Table 1. Composition and pharmacological properties of K_{ATP} channels in different tissues

Tissue	Channel composition	Inhibition by glibenclamide IC_{50}, μM	Activation by	
			diazoxide EC_{50}, μM	levcromakalim EC_{50}, μM
Pancreatic β-cell	(SUR1/ $K_{ir}6.2)_4$	0.005–0.030	20–100	>100
Cardiac myocyte	(SUR2A/ $K_{ir}6.2)_4$	0.003–0.005	>500	300
Vascular myocyte	(SUR2B/ $K_{ir}6.1)_4$	0.025	200	0.5
Skeletal muscle	(SUR2B/ $K_{ir}6.2)_4$	0.01–0.2	>500	>100
Neurons	(SUR1/ $K_{ir}6.2)_4$[1]	2.1[2]	<200	not available

From Quast [1], Quayle et al. [2], Fujita and Kurachi [3], Babenko et al. [11] and Sakura et al. [13].

[1] A novel SUR variant, SUR1B [13].

[2] Figure refers to inhibition by the sulphonylurea, tolbutamide.

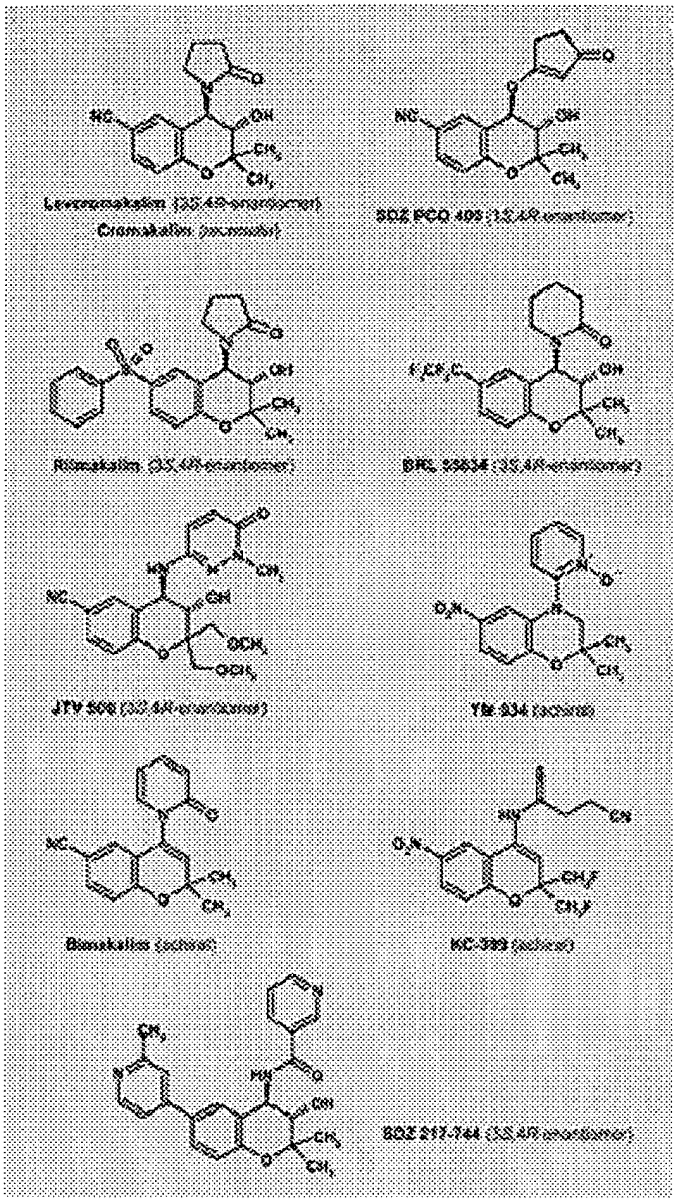

Fig. 2. KCOs profiled for asthma.

basis for their efficacy in stimulating insulin release and use in diabetes [1, 16].

Rationale for the Utility of Potassium Channel Openers in Airway Hyperreactivity

The heightened sensitivity of the airways of asthmatics to a range of bronchoconstrictor stimuli which do not usually affect normal subjects is a defining feature of asthma [17]. The phenomenon, termed bronchial (or airway) hyperreactivity (AHR), results in facilitation of bronchospasm and contributes to the airway obstruction characteristic of asthma [8, 9, 18–20]. The principal clinical symptoms of asthma, wheezing and breathlessness are a direct consequence of airway obstruction. Although the underlying mechanisms of AHR in asthma are unknown, both preclinical and clinical evidence points to an increased excitability of smooth muscle cells and/or the nervous elements of the airways as important contributory factors [18, 19]. By increasing the efflux of potassium from these cells, KCOs would induce hyperpolarization and a decrease in responsiveness to excitatory stimuli.

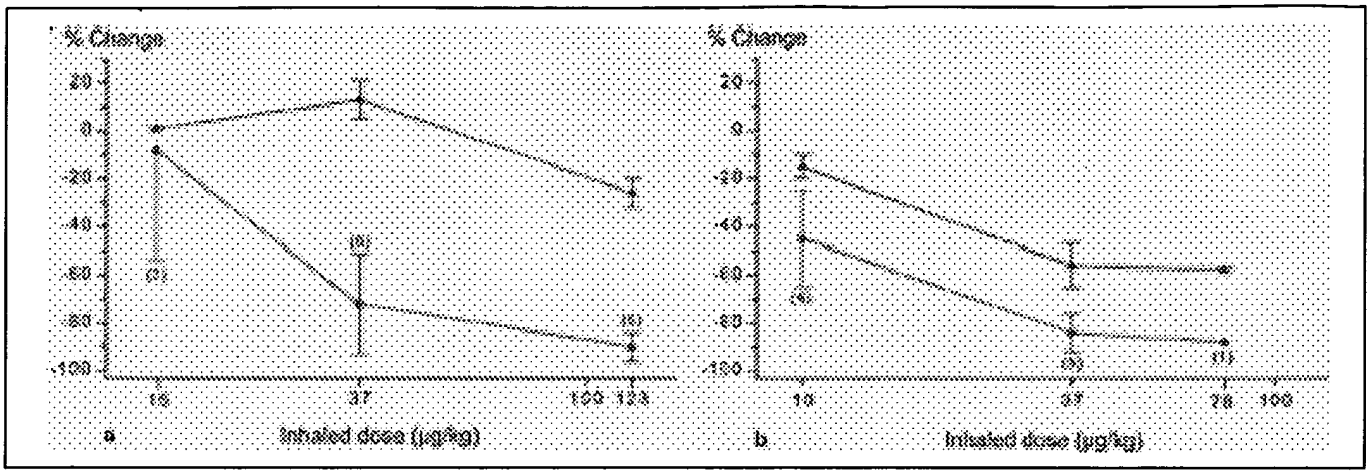

Fig. 3. Dose-response curves for the effects of SDZ 217-744 (**a**) and bimakalim (**b**) given by aerosol inhalation on the bronchoconstrictor response to inhaled methacholine (●) and diastolic blood pressure (▲) in the anaesthetized rhesus monkey. Effects were measured 5 min after the end of drug administration and are the maxima observed at each dose level. Mean values (± SEM) of the number of individual experiments shown in parentheses are presented. For further details see text.

Effects of Potassium Channel Openers in Animal Models of Airway Hyperreactivity

AHR can be induced in guinea pigs by intravenous administration of immune complexes [21, 22] or exposure to ozone [23]. Representative KCOs reverse AHR when administered locally to the airways although there are significant differences between these agents in their potencies in the two models (table 2). KCOs are significantly more potent in reversing AHR than in inducing bronchodilation in animals with normoreactive airways [20, 22]. The effects of bimakalim to suppress AHR can be blocked by pretreatment of the animals with glibenclamide, which is consistent with the involvement of K_{ATP} channels in the response [24]. The first generation KCOs, levcromakalim, bimakalim and YM 934, markedly reduce blood pressure at the doses required to inhibit AHR. In contrast, BRL 55834, JTV 506 and particularly SDZ 217-744, showed a clear separation between the two activities (table 2). Unlike salbutamol, SDZ 217-744 does not cause AHR on prolonged administration to guinea pigs; indeed, concomitant administration of SDZ 217-744 inhibits AHR induced in the same model by chronic administration of salbutamol [25]. Confirmation of the difference between first- and second-generation KCOs with respect to their therapeutic ratios for inhibiting AHR and inducing cardiovascular effects has come from experiments in rhesus monkeys (fig. 3). Thus, in animals displaying spontaneous AHR, bimakalim and SDZ 217-744 were found to be potent inhibitors of methacholine-induced bronchoconstriction.

Table 2. Comparison of potencies of benzopyran-type KCOs for their inhibition of AHR in guinea pigs induced by either immune complex (IC-AHR) or ozone (O_3-AHR) compared with their potencies in reducing mean arterial blood pressure (ΔBP)

Compound	IC-AHR ED_{50}, μg/kg	O_3-AHR ED_{50}, μg/kg	ΔBP ED_{20}, μg/kg
Levcromakalim	22	n.d.	10
Bimakalim	0.5	0.3	2
Rilmakalim	0.2	n.d.	10
JTV 506	0.5	n.d.	19
SDZ PCO 400	3.2	n.d.	30
YM 934	2.1	>100	3.4
BRL 55834	0.1	>100	10
SDZ 217-744	0.08	3	>100

From Buchheit and Hofmann [22], Yeadon et al. [23] and Buchheit [unpubl. obs.].

However, whereas bimakalim induced hypotension at similar doses, SDZ 217-744 was devoid of cardiovascular effects at doses which markedly supressed bronchoconstriction (fig. 3). Thus, a new generation of KCOs, exemplified by SDZ 217-744, is emerging with a wide therapeutic window following local administration.

Clinical Studies with Potassium Channel Openers

There have been just four clinical studies with KCOs published and all concern first generation compounds. In a study in normal volunteers, oral administration of cromakalim inhibited bronchoconstrictor responses to hista-

mine [26]. However, in a second study with levcromakalim, the active enantiomer of cromakalim, the finding could not be confirmed and headache was reported by 19 out of the 25 patients who received the drug [27]. Nevertheless, in a study in nocturnal asthma, orally administered cromakalim attenuated the fall in the early morning FEV_1; again, though, headache was a significant side effect [28]. In a further study, bimakalim showed neither bronchodilation nor cardiovascular side effects when given by inhalation to asthma patients at cumulative doses up to 175 μg [29].

Conclusions

Major recent developments in our understanding of the structural basis of the heterogeneity of K_{ATP} channels promise to reveal opportunities for novel therapies for a variety of diseases. Nowhere is this more evident than in the area of respiratory disease where a unique approach to the treatment of AHR, one of the defining characteristics of asthma, is emerging [10] supported by a wealth of preclinical evidence. The key development has been the demonstration that certain KCOs of the benzopyran class can obviate experimentally induced AHR at doses substantially below those which produce cardiovascular side effects. Since the clinical potential of earlier, first-generation KCOs was compromised by cardiovascular side effects, the new generation of compounds with selectivity for the airways may constitute a new class of drugs for the treatment of asthma.

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Monthly Focus: Central & Peripheral Nervous Systems

KCNQ potassium channels: drug targets for the treatment of epilepsy and pain

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Epilepsy and neuropathic pain are disorders characterised by excessive neuronal activity. These disorders are currently managed by drugs that are capable of dampening neuronal excitability, including voltage-gated sodium channel blockers, voltage-operated calcium channel modulators and modulators of inhibitory GABAergic neurotransmission. However, these drugs are rarely 100% efficacious and their use is often associated with limiting side effects. Thus, there is a clear medical need for novel agents to treat these diseases. One potential mechanism that has not yet been exploited is potassium (K^+) channel opening. A significant (and growing) body of genetic, molecular, physiological and pharmacological evidence now exists to indicate that KCNQ-based currents represent particularly interesting targets for the treatment of diseases such as epilepsy and neuropathic pain. Evidence supporting these K^+ channels as novel drug targets will be reviewed in the following article. Worldwide patent activity relating to KCNQ channels and KCNQ-modulating drugs and their uses will also be summarised.

Keywords: epilepsy, KCNQ2, KCNQ3, pain, potassium channel

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1. Introduction

Epilepsy and neuropathic pain are disorders characterised by either inappropriate spontaneous neuronal activity or excessive neuronal activity in response to physiological stimuli. These disorders are currently managed by drugs that are capable of dampening neuronal excitability. Drugs approved by regulatory agencies for epilepsy therapy include voltage-gated sodium channel blockers (carbamazepine, phenytoin, lamotrigine and topiramate, for example) voltage-operated calcium channel modulators (ethosuximide, gabapentin and, possibly, levetiracetam) and modulators of inhibitory GABAergic neurotransmission (benzodiazepines, vigabatrin and tiagabine, for example). Of these, gabapentin and carbamazepine are approved for the treatment of neuropathic pain and lamotrigine has demonstrated efficacy for neuropathic pain in clinical trials [1]. Although these drugs provide adequate symptom relief in many patients, a significant number of patients remain poorly treated with the currently available agents. Thus, there is a clear medical need for new drugs with novel mechanisms of action to serve as alternative or adjunct therapy for the treatment of disorders of excessive neuronal excitability.

One potential mechanism that has not yet been exploited is potassium (K^+) channel opening. K^+ channels play a major role in the control of all aspects of neuronal excitability. Activation of K^+ channels theoretically represents a powerful means of reducing excessive neuronal activity.

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2. KCNQ channels

KCNQ channels are a family of six transmembrane domain, single pore-loop, voltage-gated K^+ channels. Five members of the family have been identified to date, including the cardiac channel KCNQ1 (formerly known as KvLQT1) and four neuronal KCNQ channels, KCNQ2 – 5. In the most recently agreed nomenclature, KCNQ2 – 5 channels have been designated as Kv7.2 – 7.5, respectively [87]. For additional background information, the reader is referred to several excellent recent reviews on the KCNQ family of K^+ channels [91,92].

A significant (and growing) body of genetic, molecular, physiological and pharmacological evidence now exists to support the premise that neuronal KCNQ-based currents represent particularly interesting targets for the treatment of diseases such as epilepsy and neuropathic pain. Evidence supporting these K^+ channels as novel drug targets will be reviewed in the following article. Worldwide patent activity relating to neuronal KCNQ channels and KCNQ-modulating drugs and their uses will also be summarised.

3. KCNQ channels and epilepsy

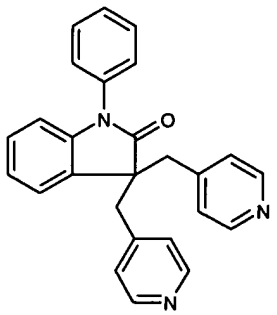
Several lines of evidence suggest that KCNQ-based K^+ currents play an important role in the control of CNS excitability and epileptogenesis.

3.1 Genetics

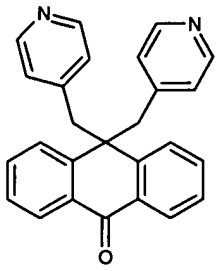
The first indication that KCNQ channels may play an important role in the control of CNS excitability came in early 1998, when groups led by Ortrud Steinlein and Mark Leppert independently identified mutations in two novel K^+ channel genes, KCNQ2 and 3, as the genetic basis of benign familial neonatal convulsions (BFNC), a rare form of neonatal epilepsy in humans [2-4]. Additional studies have now identified numerous additional mutations, particularly in KCNQ2, which are associated with BFNC [5-12]. Interestingly, the majority of the mutants described to date exhibit loss of function without dominant-negative effects and a mere 25% reduction in KCNQ2 or 3 channel function may be sufficient to cause the electrical instability in BFNC [13]. These observations indicate that KCNQ2 and 3 channels can exert a powerful stabilising influence on CNS excitability. BFNC however, is characterised by seizures in the first few weeks of postnatal life and, in most patients, symptoms spontaneously remit, possibly as a result of the postnatal maturation of the GABAergic inhibitory system and postnatal increases in KCNQ2 expression [14]. The impact of KCNQ2 and 3 mutations on the excitability of the adult human brain is difficult to assess. However, it is known that BFNC patients have an increased risk of developing seizures in later life [85]. This may suggest that the influence of KCNQ2 or 3 mutations on CNS excitability, whilst not as profound as during the neonatal period, carries through into adulthood. The possibility that reductions in KCNQ2 expression could alter CNS excitability during later developmental stages has been addressed using genetically modified mice. Watanabe and colleagues [15] produced mice with targeted deletion of the KCNQ2 gene. Homozygous mice (*Kcnq2*$^{-/-}$) died a few hours after birth as a result of pulmonary atelectasis. Heterozygous mice on the other hand, developed normally and the basal electroencephalograph (EEG) was similar to wild type (*Kcnq2*$^{+/+}$) animals. However, these mice exhibited a hypersensitivity to the convulsant pentylenetetrazol (PTZ). Yang and colleagues [16] have recently identified progeny of ethylnitrosourea (ENU)-treated C57BL/6J mice that exhibit a lowered electroconvulsive threshold. These mice were subsequently shown to harbour a single mutation, Szt1 (seizure threshold 1), consisting of a 300 kb deletion of genomic DNA involving three known genes. Two of these genes, *Kcnq2* and *Chrna4*, are known to be mutated in human epilepsy. Szt1 mice exhibit a phenotype strikingly similar to that described for *Kcnq2* knockout mice. As for *Kcnq2* null mice, homozygous Szt1 mice died shortly after birth as a result of pulmonary atelectasis and heterozygous Szt1 mice displayed an increased sensitivity to PTZ. In addition, Szt1 heterozygous mice were shown to possess a reduced electroconvulsive threshold and evidence of hippocampal abnormalities. Collectively, the available data from genetically modified mice suggests that the influence of KCNQ2 on CNS excitability is not restricted to an early developmental stage but instead, modest reductions in KCNQ2 expression appear to influence CNS excitability throughout postnatal life.

3.2 Distribution

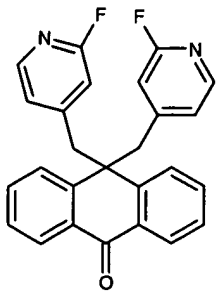
Analysis of neuronal KCNQ mRNA expression patterns reveals that these channels are expressed predominantly in the CNS. For example, overlapping expression of KCNQ2 and 3 has been observed in several areas known to be important for human epilepsy and the control of neuronal network oscillations and synchronisation, such as the cortex, thalamus and hippocampus [17-21]. KCNQ2 and 3 channels are colocalised postsynaptically on pyramidal and polymorphic neurons of the human hippocampus and cortex, where they presumably regulate excitability by controlling the integration of synaptic inputs. KCNQ2 channels may also be located presynaptically in the human hippocampus, including mossy fibre bundles and the inner dentate molecular layer, suggesting that these channels may play a role in action potential propagation and neurotransmitter release [20]. A detailed analysis revealed extensive expression of KCNQ2 throughout the mouse brain, with intense somatic expression in modulatory cholinergic and dopaminergic neurons of the basal ganglia and in neurons that regulate hippocampal excitability [21]. Recently, Devaux and colleagues [96] have also demonstrated that KCNQ2 channels are expressed in axon initial segments and nodes of Ranvier throughout the central and peripheral nervous system, where they may play a role in the control of axonal excitability. Relatively little KCNQ2 and 3 expression can be detected in peripheral organs and tissues



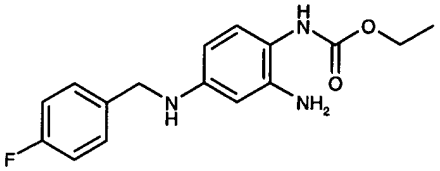
1 Linopirdine



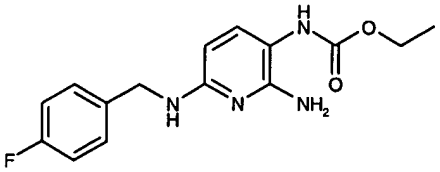
2 XE-991



3 DMP-543



4 Retigabine



5 Flupirtine

[18]. KCNQ5 also shows widespread expression in the CNS, with many regions showing overlapping expression of KCNQ2, 3 and 5 [88,89]. Intense KCNQ5 immunoreactivity was found in both pyramidal and non-pyramidal neurons and a population of glial cells throughout the temporal neocortex and the hippocampal formation of the human brain [94]. KCNQ4 on the other hand, shows restricted expression, being expressed predominantly in the outer hair cells and the nuclei of the central auditory pathway [90].

3.3 Function

The overlapping expression of KCNQ2, 3 and 5 in many brain regions suggests that these channels might normally assemble as heteromultimeric channel complexes. Indeed, coexpression of KCNQ2 and 3 in *Xenopus* oocytes leads to the generation of currents at least tenfold larger than those generated by the expression of either channel alone [18]. Furthermore, these currents exhibit very similar biophysical and pharmacological properties to a native neuronal current known to play a powerful role in the control of CNS excitability, the neuronal M-current (I_M) [19]. I_M is a non-inactivating, slowly deactivating, subthreshold current first described in sympathetic ganglia and subsequently in a variety of central neurons [19,22-25]. I_M plays an important role in controlling resting membrane potential, responsiveness to synaptic inputs and spike frequency adaptation. I_M is also regulated by neurotransmitters and neuropeptides and I_M inhibition, at least in part, mediates muscarinic and peptidergic excitation of central neurons [86]. KCNQ5 and 3 also form functional heteromeric channels in *Xenopus* oocytes [88,89], suggesting that heterotetramers of KCNQ5 and 3 may also make a significant contribution to the I_M in some brain regions [91]. The finding that heteromeric KCNQ channels represent the molecular correlate of I_M, strongly supports a role for these channels in the control of CNS excitability. These findings also suggest that KCNQ channels, especially as heteromeric complexes, represent promising targets for drugs designed to alter neuronal excitability.

3.4 Pharmacology

3.4.1 Pharmacological agents for the study of KCNQ channels

A number of pharmacological tools have been identified which modulate KCNQ and/or M-currents. The first selective I_M inhibitor described was linopirdine (compound 1). This compound blocks neuronal M-currents at concentrations that are without effect on other neuronal K^+ currents [26-28]. Linopirdine also blocks KCNQ channels at concentrations that are without effect on related K^+ channels [19,93]. Related compounds include XE-991 (compound 2) and DMP-543 (compound 3) [29,30].

Retigabine (compound 4, N-(2-amino-4-[fluorobenzylamino]-phenyl) carbamic acid, D-23129) and flupirtine (compound 5) are KCNQ and I_M activators. Retigabine was first identified as a K^+ channel opener by Chris Rundfeldt [31,32], who showed that this drug could increase barium and weakly tetraethylammonium sensitive, 4-aminopyridine-insensitive K^+ conductance in NG108-15, hNT, PC12 cells and isolated mouse cortical neurons. In addition, retigabine has been shown to induce membrane hyperpolarisation in neurons in rat hippocampal–entorhinal cortex slices [33]. Until recently, the molecular nature of the K^+ channel opened by retigabine was unknown. In 2000 however, three groups independently identified KCNQ2/3 channels as a molecular target for retigabine [34-36]. Data generated by these groups demonstrated that retigabine, at concentrations between 0.1 and 10 μM, enhanced KCNQ2/3 currents by inducing profound leftward shifts in the voltage-dependence

of channel activation. Additional studies have demonstrated that retigabine appears to be a relatively non-selective KCNQ channel modulator, being capable of activating all members of the KCNQ channel family except KCNQ1 [37,93]. Retigabine also enhances native M-currents in PC-12 cells, rat sympathetic neurons and rat dorsal root ganglion cells [36-38]. Evidence also exists to suggest that retigabine may possess additional actions. For example, retigabine has been shown to increase the synthesis of GABA in rat hippocampal slices and to enhance GABA-induced chloride currents in cultured rat cortical neurons [39,40]. Retigabine may also possess weak sodium and calcium channel blocking activity [40]. However, these effects are generally only seen at concentrations 10 – 100-fold higher than those required for KCNQ activation [41]. It seems therefore likely, that KCNQ activation represents the primary pharmacological action of retigabine.

In addition to retigabine, recent preliminary data suggests that a structurally related compound, flupirtine, also activates KCNQ2/3 channels and native M-currents. Although flupirtine is generally considered to be either an NMDA antagonist or an α_2-adrenergic agonist, this is based only on indirect *in vitro* and *in vivo* data. Direct evidence for these mechanisms is noticeably lacking. On the contrary, direct evidence has now emerged to suggest that flupirtine, like retigabine, possesses the ability to enhance the activation of KCNQ2/3 K^+ channels. Using whole-cell patch clamp recordings from HEK293 cells transiently transfected with KCNQ2/3 constructs, Ilyin and colleagues [42] determined that flupirtine (10 µM) was a positive modulator of KCNQ channels with a mechanism of action similar to that of retigabine. Flupirtine increased current amplitude, caused a hyperpolarising shift in the KCNQ2/3 activation curve and slowed KCNQ2/3 deactivation kinetics.

3.4.2 *KCNQ modulators in* in vitro *and* in vivo *models of epilepsy*

The agents described in the preceding section (linopirdine, XE-991, retigabine and flupirtine) have been used to help define the role of KCNQ channels and M-currents in the control of CNS excitability. KCNQ blockers, such as linopirdine and XE-991, appear to increase neuronal excitability as evidenced by their ability to increase neurotransmitter release and enhance cognition in a variety of animal models [43-45]. Furthermore, the KCNQ K^+ channel inhibitor linopirdine (30 mg/kg) is proconvulsant [46].

Retigabine and, to a lesser extent, flupirtine have been evaluated in animal models of epilepsy. Consistent with the widespread distribution of KCNQ channels and the important role played by these channels, retigabine exerts anticonvulsant activity in a broad range of seizure models. Retigabine prevents epileptiform activity induced by 4-aminopyridine, bicuculline, low magensium (Mg^{2+}) and low calcium (Ca^{2+}) in hippocampal slices [47,48] and seizures induced by PTZ, maximal electroshock, kainate, penicillin, picrotoxin and NMDA in rodents [49-51]. Retigabine is also effective against audiogenic seizures in DBA/2J mice, against seizures in epilepsy-prone rats and against seizures in an amygdala-kindling model [51-53]. Flupirtine is effective against PTZ-induced seizures in mice [54].

3.5 Clinical

Further support for the validity of KCNQ channels as targets for novel antiepileptic agents comes from clinical studies with retigabine and flupirtine. Retigabine is being developed for the treatment of epilepsy and has been evaluated in five Phase IIa (efficacy and dose-range-finding) clinical trials as well as a long-term extension study [55]. In two add-on, open-label, studies in patients with treatment-resistant partial seizures (> 4 seizures/month), 12 out of 35 patients completing the studies showed > 50% reduction in seizure frequency [56]. In a larger, randomised, double-blind, placebo-controlled dose-ranging add-on study in 399 patients, retigabine at 900 and 1200 mg resulted in statistically significant reductions in seizures. Median reductions of 13, 23, 29 and 35% for placebo and daily doses of 600, 900 and 1200 mg, respectively, were observed. It was found that 16, 23, 32 and 33% of patients exhibited a 50% or greater reduction in seizures (ie. the responder population) following daily doses of placebo, 600, 900 and 1200 mg, respectively [55]. In pharmocokinetic studies, plasma concentrations reached ~ 5 µM at a minimally effective dose of retigabine (350 mg b.i.d.) [57]. These concentrations are very similar to those required for KCNQ channel opening, supporting the involvement of these channels in the anticonvulsant effects of retigabine.

Flupirtine has been evaluated in a small-scale clinical trial involving 4 patients with refractory epilepsy. Flupirtine (400 mg/day) was administered in conjunction with existing antiepileptic therapy and all 4 patients showed a decrease in seizure frequency [54]. In a second trial, 400 – 800 mg/day flupirtine, administered with existing therapy, reduced seizure frequency in eight out of nine patients [58].

In addition to providing information on the possibility that activation of KCNQ channels can be beneficial for the treatment of epilepsy, clinical studies can provide information regarding unwanted adverse effects associated with KCNQ activation. In healthy volunteers administered increasing doses of retigabine, dizziness was the most frequent adverse event and occurred in a dose-related manner [57]. Dizziness was not reported by any subjects receiving placebo. Dose-limiting adverse events observed at 600 mg retigabine were chills, pain, symptomatic hypotension, dizziness, nausea, myalgia, sweating and vomiting. Asthenia and somnolence were also reported in the study. In patients administered retigabine as add-on therapy to their existing antiepileptic drugs, the most common adverse events were asthenia, dizziness, headache, somnolence, tremor, speech disorder, amnesia, ataxia, blurred vision, mental slowing and vertigo [55,56].

4. KCNQ channels and neuropathic pain

4.1 Distribution

Painful stimuli are transferred to the CNS by the lateral spinothalamic tract. First order neurons transmitting pain impulses from the skin (Aδ and C fibres) enter the substantia gelatinosa of the dorsal horn via the dorsal roots. Second order neurons in the lateral spinothalamic tracts convey impulses associated with pain up to the nuclei of the ventroposterior thalamus (ventroposterior medial nucleus [VPM] and ventroposterior lateral nucleus [VPL]) where the painful impulses are integrated. From the thalamus, third order neurons convey the impulses up to the cerebral cortex, where subjective interpretation of pain is thought to occur. Additional background information on pain pathways and processing can be found in an excellent review article by Hunt and Mantyh [98]. Additional information concerning pain terminology can be found at http://www.iasp-pain.org/pubsopen.html [201].

KCNQ2, 3 and 5 mRNA is expressed at key locations in the pain pathway. Expression of KCNQ2 and 3 has been identified in the thalamus, including the ventroposterior thalamus, the cerebral cortex and the dorsal and ventral spinal cord [17-19,21,97]. In the periphery, KCNQ2, 3 and 5 mRNA can be found in first order sensory neurons in the isolated rat dorsal root ganglia [38,59]. In addition, using immunofluorescence and electrophysiological techniques, David Brown's group has shown that KCNQ2, 3 and 5 protein and M-currents are expressed in cells of the rat DRG. Immunofluorescence studies using selective antibodies identified expression of these subunits in the somata and neuronal processes of both small and large diameter DRG neurons. Many cells coexpressed more than one KCNQ subunit. Electrophysiological experiments revealed the presence of linopirdine and retigabine-sensitive M-currents in small and large diameter DRG neurons and many of the small cells that possessed M-currents also responded to capsaicin, identifying them as nociceptive neurons [38]. In addition, as noted in Section 3.2, KCNQ2 channels are expressed in axon initial segments and nodes of Ranvier in the peripheral nervous system [96]. The finding that KCNQ channels are expressed in peripheral sensory nerves suggests that, in addition to playing a role in central pain perception, KCNQ channels may also play a role in peripheral pain processing.

4.2 Pharmacology

Direct evidence supporting a role for KCNQ channels in the control of sensory nerve excitability and pain processing comes predominantly from pharmacological studies. In electrophysiological studies, the KCNQ activator retigabine, has been shown to hyperpolarise isolated small diameter DRG cells (nociceptive neurons) and increase the firing threshold. This effect could be blocked by the KCNQ blockers linopirdine and XE-991 [38]. Passmore and colleagues [38] also studied the effects of retigabine on electrically and naturally (thermal and mechanical) evoked neuronal responses in dorsal horn neurons of both naive rats and rats that had previously undergone L4 and L5 spinal nerve ligation. Retigabine produced a statistically significant, dose-related inhibition of firing in small-diameter C and Aδ nociceptive neurons in both naive and ligated animals. Interestingly, retigabine also seemed highly effective at inhibiting indices of neuronal hyperexcitability such as postdischarge spikes and 'wind-up' in dorsal horn neurons, possibly indicative of an enhanced role for KCNQ channels in hyperexcitable neurons. Indeed, preliminary observations suggest that KCNQ2, 3 and 5 expression in the dorsal root ganglion appears to be maintained following nerve injury, whereas expression of other K^+ channels may be decreased (compare [59] with [60-63]).

Recently, Rivera-Arconada and colleagues have also shown that retigabine could depress responses to activation of nociceptive afferent fibres in a rat hemisected spinal cord preparation, whereas XE-991 showed the opposite effect and reversed effects of retigabine [95]. Interestingly, KCNQ modulators exerted only weak effects on non-nociceptive reflexes [95]. Finally, retigabine has been shown to delay the time to peak and reduce the amplitude of the compound action potential in the rat sciatic nerve in a linopirdine-sensitive manner [96].

Collectively, the observations described above clearly support the contention that KCNQ channels and corresponding M-currents may represent novel targets for the treatment of pain. In particular, these channels may represent targets for the treatment of pain states associated with hyperexcitability of peripheral sensory nerves, such as neuropathic and inflammatory pain.

Behavioural studies have also provided evidence to support a role for KCNQ channels in pain perception. Blackburn-Munro and Jensen [64] tested retigabine in several rat models of nociceptive, persistant and chronic pain. In the chronic constriction injury model of neuropathic pain, retigabine attenuated mechanical hypersensitivity to pin prick stimulation and cold allodynia but had no effect on tactile allodynia when measured using von Frey hairs. Attenuation of hyperalgesia to a pin-prick response was also observed with retigabine in the spared nerve model of neuropathic pain but again this compound produced no antiallodynic effect when measured using von Frey hairs. In the formalin model of persistant pain, retigabine attenuated Phase II flinching (a response thought to result from secondary spinal sensitisation) and this effect could be completely reversed by the KCNQ channel blocker XE-991. Acute pain, as measured by Phase I flinching in the formalin test and in the tail flick model, was unaffected by retigabine or XE-991. Importantly, retigabine did not appear to impair motor coordination at the doses tested in the pain models described above. The attenuation of behavioural responses to painful stimuli therefore appears to represent a genuine antinociceptive property of retigabine.

Rostock and colleagues [65] tested retigabine against the thermal hyperalgesia produced by either the ligation or transection of the L5 spinal nerve. In both models, retigabine displayed antihyperalgesic activity as evidenced by

increased paw withdrawal latencies to thermal stimuli. The same authors also tested retigabine in the formalin model and like Blackburn-Munro and Jensen [64], found no activity against Phase I flinching but a significant reduction in Phase II flinching.

In a third study, Passmore and colleagues [38] tested retigabine in the rat carrageenan model of inflammatory pain. Weight distribution was used as a measure of nociception following intraplantar administration of 2% carrageenan. Carrageenan injection produced a significant redistribution of weight bearing such that the inflamed paw bore only 21% of the hind paw load (normal weight bearing would be 50% on each hind paw). Administration of retigabine increased the weight born by the inflamed paw up to 41%, an effect that was blocked by coadministration of XE-991.

As noted above, flupirtine, a close analogue of retigabine also modulates KCNQ channels with a similar mechanism of action to that of retigabine [42]. In animal pain models flupirtine is active in the rat tail flick assay [66] and in the spinal nerve ligation model of neuropathic pain [42], providing further support for the role of KCNQ channels in pain processing and perception.

4.3 Clinical

The validity of KCNQ channels as targets for novel analgesic agents is supported by extensive clinical experience with flupirtine. Flupirtine has been marketed in Europe since 1984 as an analgesic under the trade name Katadolon® (Asta Medica). Flupirtine has been shown to be effective for the relief of many types of pain. In double-blind, placebo- or active-controlled studies, flupirtine was efficacious for the treatment of tumour or cancer pain [67-69], for postoperative pain [70-73], for pain following episiotomy [72] and for trauma pain [72]. Doses varied between 100 mg and 300 mg per single dose with administration of up to six doses (usually three) per day. Most studies were conducted using oral administration, but flupirtine was occasionally administered as a suppository.

Flupirtine was effective after a single dose [72] and was effective for up to 1 year of treatment with no evidence of development of tolerance [74]. For tumour/cancer pain, flupirtine efficacy was superior to tramadol, an analgesic with opiate and monaminergic mechanisms of action [75] and pentazocine [69]. In studies of postoperative pain, flupirtine was as effective as pentazocine and dihydrocodeine as well as metamizole, paracetamol and naproxen [71], it was also superior to placebo (69% pain reduction versus 26%) [72]. For postepisiotomy pain, flupirtine was superior to suprofen and for post-traumatic sports injury pain it was superior to paracetamol plus massage. In one controlled trial in migraine patients, there was a trend for flupirtine efficacy [76].

Flupirtine administration was not associated with a high incidence of adverse effects in clinical trials and had fewer adverse effects than the active control drugs, pentazocine and dihydrocodeine [71]. The most common adverse events associated with flupirtine administration in patients were drowsiness, dizziness, dry mouth, pruritis and nausea [72,74,77].

In summary, flupirtine alleviates pain following acute administration without development of tolerance in a variety of painful conditions, including severe pain (cancer and postoperative pain). Low micromolar plasma concentrations (2.5 – 6.5 μM) of flupirtine were associated with doses providing analgesic activity in humans [78]. The only pharmacological activity known to occur in this concentration range for flupirtine is activation of K^+ channels [42,79,80]. Thus, while the definitive mechanism of action for flupirtine remains to be resolved, activation of KCNQ channels seems a reasonable hypothesis.

5. Summary of patent activity

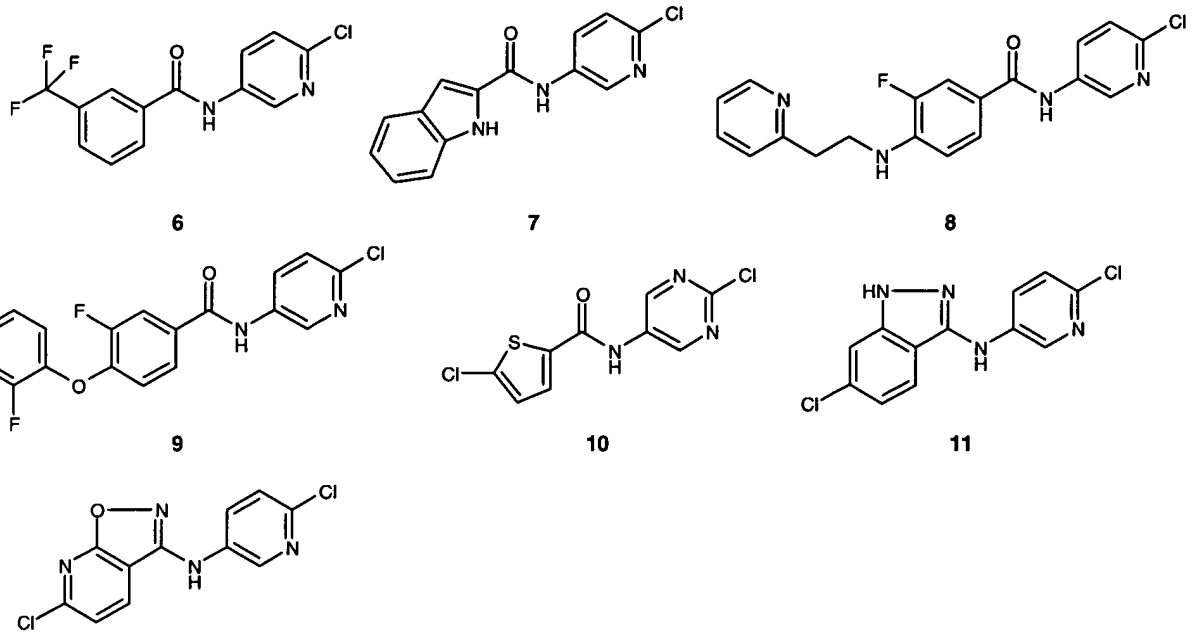
5.1 Patents relating KCNQ2, KCNQ3 and KCNQ5 channels

At the time of writing, three US patents had been issued covering KCNQ2 and/or KCNQ3 and uses thereof. In US6403360, Bristol-Myers Squibb (BMS) describes the nucleic and amino acid sequence of human, rat and mouse KCNQ2 and human KCNQ3 and the distribution of these genes in tissues, regions of the brain and the spinal cord. US6403360 also describes the function and preliminary pharmacology of human, rat and mouse KCNQ2 and human KCNQ3 when expressed in *Xenopus* oocytes [101]. In the second patent issued (US6413719), University of Utah Research Foundation describes human KCNQ2 and 3 genes, the nucleic and amino acid sequences of human and mouse KCNQ2 and 3 and the identification of KCNQ2 and 3 mutations in patients with benign familial neonatal convulsions [102]. Finally, in US6472165, Arzneimittelwerk Dresden GmbH present data showing that retigabine is a selective KCNQ2 and 3 activator and describe possible methods for identifying compounds that modulate KCNQ2 or KCNQ2/3 that involve expressing these channel subunits in a cell and either comparing the effect of a substance to that of retigabine or by determining whether a substance is capable of competing with retigabine [103].

Two additional US patents have been issued covering KCNQ5 genes. US6617131 (Aventis Pharma Deutschland GmbH) describes the nucleic and amino acid sequence of human KCNQ5, its distribution in a variety of human tissues and a preliminary evaluation of the function and pharmacology of this gene [123]. US6649371 (Neurosearch A/S) also describes the function and pharmacology of human KCNQ5 [124].

5.2 Patents relating KCNQ activation to potential therapeutic targets

The potential role of KCNQ channel activators in reducing neuronal excitability in tissues expressing these channels has led to a plethora of applications and patents covering their use for a wide range of diseases and conditions. Icagen was the first to address the potential of KCNQ activators to effectively modulate pain states and was granted a patent outlining a method for reducing pain in a subject by increasing ion flow



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through KCNQ K^+ channels. Selective KCNQ activators were shown to produce dose-dependent analgesic effects in the mouse formalin model [104]. In 2002, BMS published an application describing the use of activators of CNS-associated KCNQ channels for the treatment of migraine or migraine-related disorders [105]. Supporting data focused on the ability of KCNQ compounds from three different series (given i.v.) to reduce superior sagital sinus-stimulated trigeminal field potentials. American Home Products (Wyeth) was recently granted a patent that described KCNQ activators for the use of maintaining bladder control and/or treatment of urinary incontinence [106]. In this patent, KCNQ channels were shown to be expressed in the bladder. Furthermore, retigabine was shown to elicit a concentration-dependent inhibition of KCl-stimulated contractions of isolated rat bladder strips and completely inhibited acetic acid-induced micturition in rats after a 10 mg/kg intraperitoneal dose. Wyeth published another application that outlined the use of KCNQ activators for the treatment or inhibition of hyperactive gastric motility. This claim was supported by the finding that retigabine produced a concentration-dependent inhibition of both KCl- and carbachol-induced ileal contractions [107]. Further experiments showed that the KCNQ blocker XE-991 reversed the retigabine effect.

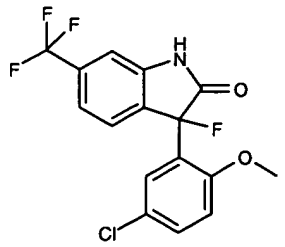
5.3 Patents claiming the use of retigabine for non-epileptic conditions

Retigabine (compound 4), a 1,2,4-triaminosubsituted-benzene compound, was found to be an effective anticonvulsant via a traditional *in vivo* screening campaign. Its activity is now believed to be due, in part, to its ability to open KCNQ channels [34-36]. More recently, the closely related pyridine analogue, flupirtine (compound 5), has also been shown to be an effective KCNQ2/3 agonist [42]. The potential use of retigabine (compound 4) for the treatment of a wide variety of disorders related to neuronal excitability has been the basis of several utility patents and patent applications. Wyeth were recently granted a patent which includes the use of retigabine and close analogues for the treatment of anxiety, while Asta Medica have patents claiming the use of retigabine for the treatment of neuropathic pain (retigabine produced a dose-dependent reduction in formalin-induced hyperalgesia), neurodegenerative disorders and for the treatment of chronic reduced cerebral blood supply (retigabine was shown to increase learning ability after constriction of cerebral blood supply in rats) [108-111]. The Glaxo group has an application describing the use of retigabine for the treatment of a number of disorders including cognitive disorders, cancerous diseases, inflammatory processes and ophthalmic diseases, however, no supporting data were reported [112].

5.4 Patents describing new KCNQ activators

Since the identification of the KCNQ channel family and their potential therapeutic utility, several programmes to identify selective KCNQ channel agonists using rational drug design techniques have been initiated. During the past 4 years, BMS, Neurosearch and Icagen have disclosed compounds that activate KCNQ channels.

The first series of agonists to be published was a collection of unique 6-substituted-pyridin-3-yl-based benzamides and related heterocyclic amides from Icagen, compounds 6 and 7 being representative examples [104,113,114]. Further explorations around the phenyl moiety of the benzamide compounds led to the identification of numerous additional analogues, such as compounds 8 and 9 [115]. A subsequent patent application discloses 2-substituted-pyrimidin-5-yl compounds (such as compound 10) [116]. The utilisation of several different conformational constraints/amide bond bioisosteres to the Icagen benzamides generated



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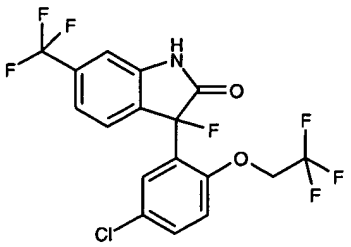

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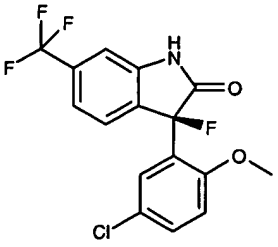

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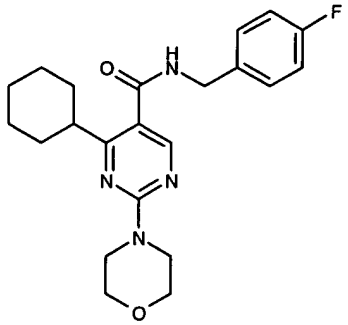

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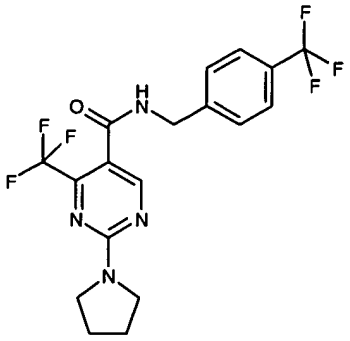

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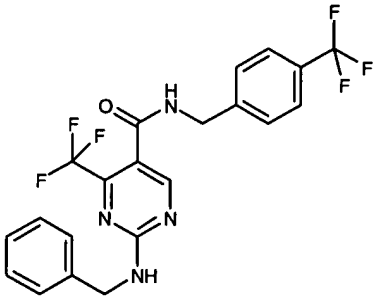

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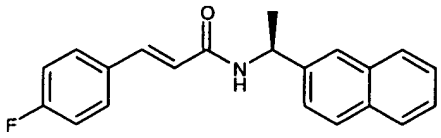

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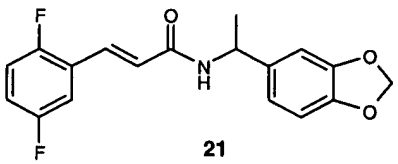

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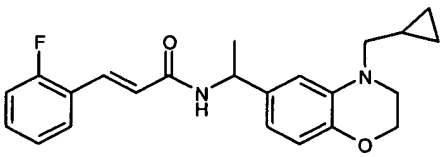

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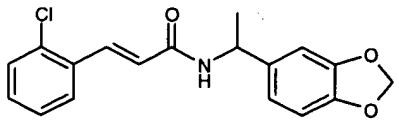

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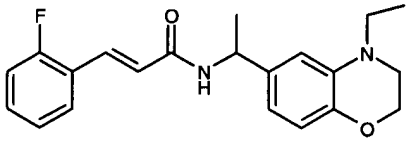

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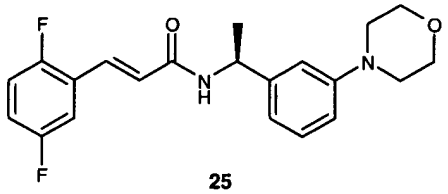

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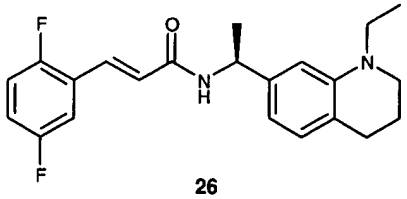

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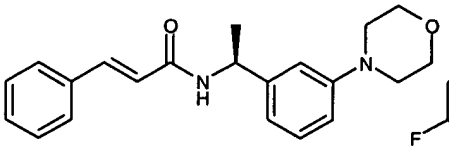

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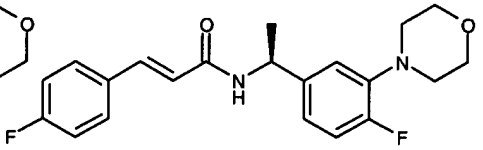

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bicyclic KCNQ agonists, such as the indazoles (compound 11) and benzisoxazoles (compound 12) [117].

Scientists at Neurosearch identified the racemic fluorooxindole (compound 13) as an agonist of KCNQ family members KCNQ4 (effective concentration for half-maximum response [EC_{50}] value = 2.4 μM) and KCNQ5 and filed an application regarding the use of this chemical class for a variety of conditions [81,122]. Both the racemate (compound 14) and its (+)-enantiomer (compound 15) were found to increase current flow through murine KCNQ2 channels at 10 μM [82,118,119]. Interestingly, the (+)-enantiomer of compound 13, compound 16, has already been in clinical trials based upon its maxi-K activity.

BMS have reported two other classes of small molecule agonists during the last 2 years. The 2,4-disubstituted pyrimidine-5-carboxamide agonists are exemplified by compounds 17 – 19 [120]. The trifluoromethoxy compound 18 increased current flow through murine KCNQ2 channels when tested

at 5 μM. Compound 18 also caused a dose-dependent reduction in superior sagital sinus stimulated trigeminal field response model of migraine. The second series of compounds were the cinnamide derivatives [121]. Compounds 20 – 22 possessed EC_{50} values against murine KCNQ2 channels in the micromolar range, whereas the structurally similar compounds 23 and 24 were considerably more potent with EC_{50} values of 0.6 and 0.9 nM, respectively. A comparison of compounds 21, 25 and 26 revealed that structurally and electronically different aryl systems did not significantly affect potency. Several different electron-modulating groups were tolerated on the cinnamide aryl. Small halogens, such as fluoro and chloro, provided good activity and presumably greater *in vivo* stability. Interestingly, the switch between 2,5-difluorophenyl (compound 21) and 2-chlorophenyl (compound 23) resulted in an almost 1000-fold increase in potency.

A number of the BMS compounds were tested in animal models of migraine, anxiety, neuropathic pain and others. When given intravenously (typically 1 mg/kg) the test compounds caused a reduction in the number of spreading depressions comparable to the effect observed with 100 mg/kg of intravenous valproic acid in a rat cortical spreading depression model. Compounds were also shown to be reasonably efficacious in rat models of neuropathic pain (Chung and Streptozotocin models), however higher doses were generally required (typically 10 mg/kg, i.v.). More information regarding the *in vitro* and *in vivo* development of this chemical class of compounds has been recently published. Compound 27 was found to open murine KCNQ2 channels in single cell voltage-clamp experiments, with an EC_{50} value of 3.3 μM and it hyperpolarised the membrane potential in SH-SY5Y human neuroblastoma cells expressing endogenous KCNQ channels, with an EC_{50} value of 0.69 μM. Compound 27 also exhibited good oral bioavailability in both rats and dogs and doses of 30 and 10 mg/kg (intragastric) of compound 26 were found to be efficacious in a rat model of migraine [83]. Unfortunately compound 27 suffered from undesired CYP450 interactions. The selectively fluorinated analogue 28 however, maintained good potency against the murine and endogenously expressed human KCNQ2 channels (EC_{50} values of 1.2 and 1.55 μM, respectively) and was devoid of these unwanted cytochrome P450 interactions [84].

6. Expert opinion

The findings described above provide a high level of validation for KCNQ channels as antiepileptic and analgesic drug targets. However, it should be noted that pharmacological studies with retigabine and flupirtine are central to the argument that KCNQ channels represent valid therapeutic drug targets and, as noted above, these drugs may possess actions unrelated to KCNQ opening. For example, retigabine has been shown to enhance GABAergic transmission in the CNS. Similarly, it has been proposed that flupirtine may interact with NMDA and α2-adrenergic receptors. It is unclear, therefore, if the efficacy of retigabine and flupirtine in animals models of epilepsy and pain and in human studies is entirely due to KCNQ activation. Final validation of KCNQ channels as antiepileptic and analgesic drug targets therefore, will require the identification of novel, highly selective KCNQ openers. *In vivo* data with novel, selective KCNQ activators is eagerly awaited and should provide the final piece of evidence to unequivocally validate KCNQ as therapeutic drug targets for the treatment of epilepsy and pain.

Disclaimer

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Pharmacological characterisation of acid-induced muscle allodynia in rats

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Abstract

Previous studies have shown that repeated injections of acidic saline, given into the lateral gastrocnemius muscle of rats, results in a bilateral reduction in withdrawal threshold to tactile stimulation of the hindpaws. We have now characterised this model of muscoskeletal pain pharmacologically, by evaluating the antinociceptive effects of various analgesics after systemic administration. The μ-opioid receptor agonist morphine (3 and 6 mg/kg) produced a particularly prolonged antiallodynic effect. The glutamate receptor antagonists ([8-methyl-5-(4-(*N*,*N*-dimethylsulfamoyl)phenyl)-6,7,8,9,-tetrahydro-1H-pyrrolo[3,2-*h*]-iso-quinoline-2,3-dione-3-*O*-(4-hydroxybutyric acid-2-yl)oxime] NS1209 and ketamine (6 and 15 mg/kg, respectively), the KCNQ K^+ channel openers retigabine and flupirtine (10 and 20 mg/kg, respectively) and the Na^+ channel blocker mexiletine (37.5 mg/kg) also significantly increased paw withdrawal threshold, although to a lesser degree than morphine. In contrast, the anticonvulsant lamotrigine (30 mg/kg), the cyclooxygenase-2 inhibitor carprofen (15 mg/kg) and the benzodiazepine diazepam (3 mg/kg) were ineffective. All antinociceptive effects were observed at nonataxic doses as determined by the rotarod test. These results suggest that in this model, muscle-mediated pain can be alleviated by various analgesics with differing mechanisms of action, and that once established ongoing inflammation does not appear to contribute to this process.

Keywords: Central sensitisation; Fibromyalgia; GABA (gamma aminobutyric acid); Glutamate receptor antagonist; Muscle pain; Na^- channel blockers

1. Introduction

Long-term changes in activity and function of nociceptive pain pathways can occur as a result of inflammatory- or neuropathic-mediated damage within both the peripheral and central nervous systems (Woolf and Salter, 2000). A consequence of this can be the expression of accentuated pain-related behaviours, reflecting hyperalgesia and allodynia in response to thermal and mechanical stimulation. Pain associated with muscoskeletal conditions often induce disability and represent a significant financial burden to society in terms of health care costs and lost productivity (Yelin and Callahan, 1995). Chronic pain conditions such as fibromyalgia are characterised by widespread muscle pain and joint tenderness (Wolfe et al., 1990). A recent report of enhanced temporal summation (wind up) in fibromyalgia patients, consistent with central sensitisation (Price et al., 2002), suggests that the aetiological development of chronic musculoskeletal pain in humans may share a number of common underlying mechanisms with other chronic pain conditions including those of neuropathic origin (Zimmermann, 1991). Similar to neuropathic pain in humans, chronic muscoskeletal pain conditions remain somewhat refractory to treatment with currently available analgesics, reinforcing the need to develop appropriate animal models of muscle-associated chronic pain.

Recently, Sluka et al. have developed a model of acid-induced pain in rodents, which has been suggested to have greater face validity to pain of muscoskeletal origin in humans. In this model, two injections of acidic (pH 4) saline separated by 2–5 days, given into the gastrocnemius muscle, have been shown to produce a long-lasting bilateral decrease in hindpaw withdrawal threshold as evaluated by von Frey hair stimulation (Sluka et al., 2001). Morphological analysis of the injected muscle revealed no obvious muscle damage associated with acidic saline injection, whilst injection of lidocaine into the ipsilateral muscle attenuated nociceptive scores of the ipsilateral hindpaw only (Sluka et al., 2001). This latter observation, together with the more recent demonstration that intrathecal injection of

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either glutamate receptor antagonists or opioid receptor agonists attenuates hindpaw hypersensitivity to sensory stimulation, suggests that the maintenance of acid-induced chronic pain in this model is also centrally mediated (Skyba et al., 2002).

In order to provide a thorough pharmacological characterisation of this model of acid-induced chronic pain in the rat, various drugs including morphine, glutamate receptor antagonists, voltage-activated Na^+ channel blockers, KCNQ K^+ channel openers (Rogawski, 2000), carprofen and diazepam, all of which target specific aspects of nociceptive transmission, were administered systemically and the effects on pain-like behaviours observed.

2. Materials and methods

Adult male Sprague–Dawley rats (Möllegaard, Denmark) weighing 200–300 g on the day of the first acidic saline injection were used in this study. The animals were housed on soft bedding, two per cage, with food and water ad libitum. The light–dark cycle was 12:12 h. The experiments were performed according to the Ethical Guidelines of the International Association for the Study of Pain (Zimmermann, 1983) and the Danish Committee for Experiments on animals.

2.1. Injection of the muscle

The method of inducing muscle-mediated chronic pain has been described in detail previously (Sluka et al., 2001). The skin covering the lateral gastrocnemius muscle was shaved, and rats were briefly anesthetized with a gaseous mixture of isoflurane (3–5%) supplemented with oxygen. One lateral gastrocnemius muscle was injected with 100 μl of HCl-adjusted preservative-free physiological saline (9 g NaCl/l; pH 4.0 ± 0.1). Five days later the same gastrocnemius muscle was re-injected using an identical injection protocol. As a control for the injection procedure itself, a separate group of eight animals were injected with saline at pH 7.4.

2.2. Behavioural testing

For testing of mechanical allodynia, mechanical hyperalgesia and cold allodynia, the animals were placed on an elevated metal grid allowing stimulation of the plantar surface of the paw, and the animals were allowed to adapt to their environment for 15 min.

2.2.1. Mechanical allodynia

The presence of mechanical allodynia was assessed using a series of von Frey nylon hairs (0.94–19.4 g) (Stoelting, IL, USA), which were applied in increasing force until the rat withdrew its hindpaw. Each hair was applied five times and the threshold (g) was taken as the lowest force that caused at least three withdrawals out of the five consecutive stimuli (Erichsen and Blackburn-Munro, 2002). Von Frey nylon hairs were calibrated both prior to and throughout the time course of the entire study to ensure that consistent bending forces were routinely applied.

2.2.2. Mechanical hyperalgesia

A pin prick test was used to test for the presence of mechanical hyperalgesia (Blackburn-Munro and Jensen, 2003). The plantar surface of the hindpaw was pressured with the point of a safety pin at an intensity insufficient to penetrate the skin, and the duration of the withdrawal response recorded with a stopwatch.

2.2.3. Cold allodynia

To test for the presence of cold hypersensitivity, ethyl chloride (Perstorps, Sweden) was sprayed onto the plantar surface of the hindpaw and animals were observed for both the intensity of the response and any paw withdrawal duration (Blackburn-Munro and Jensen, 2003). This was then classified according to the following scale: 0—no visible response; 1—startle response without paw withdrawal; 2—clear withdrawal of the paw for (0–1 s); 3—prolonged withdrawal (1–5 s) often combined with flinching and licking of the paw; 4—prolonged repetitive withdrawal (>5 s) and/or vocalization.

2.2.4. Thermal hyperalgesia

Finally, to test for the presence of thermal hyperalgesia, reflex nociceptive pain was assessed using the rat plantar test (Ugo Basile, Comerio, Italy) following a modified method of Hargreaves et al. (1988). Single rats were placed in individual perspex boxes on a glass platform and allowed to habituate for 15 min. A mobile radiant heat source was located under the platform and focussed onto the plantar surface of each hindpaw in turn, enabling paw withdrawal latency values to be recorded. The thermal hyperalgesia response was measured twice before acid injection and then on days 2, 7, 11 and 14 post-injection.

All other behavioural responses to sensory stimulation of the hindpaw described above were assessed before the first intramuscular (i.m.) acid injection and then at 3 and 24 h later. They were then measured again immediately before the second i.m. acid injection and again at 3 and 24 h later. Thereafter, they were measured routinely each week for up to 5 weeks. Only animals with a paw withdrawal threshold to von Frey hair stimulation of 5.41 g or less on the day of the experiment were included for drug testing. At least 7 days after the second injection of acid, either drug or vehicle was injected and behavioural responses measured by an experimenter blinded to treatment.

2.3. Rotarod testing for ataxia

In normal rats changes in motor performance after drug administration were evaluated using the rotarod test (with

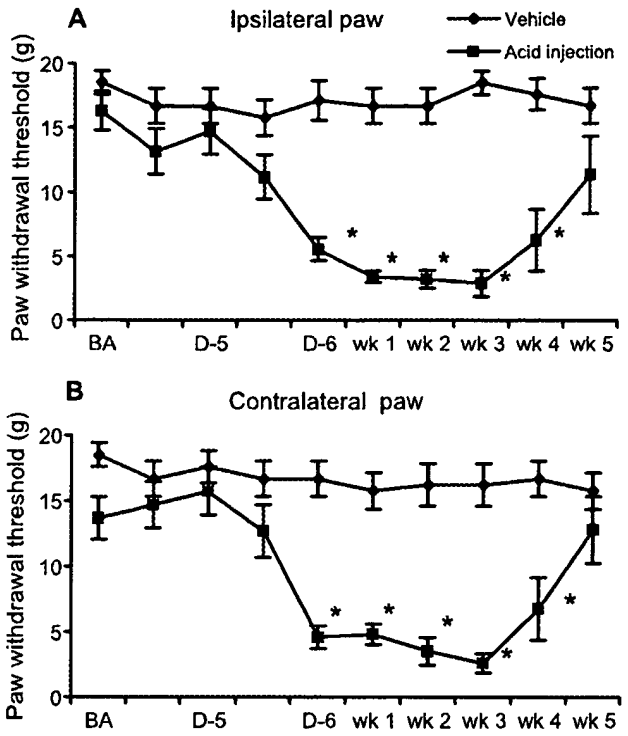

Fig. 1. Sensitivity changes of the hindpaw after repeated injections of acidic saline in the gastrocnemius muscle, in 72 % of the animals injected. Animals that did not develop allodynia were excluded from the study. Withdrawal threshold (g) after stimulation of the plantar surface of the hindpaw was measured with von Frey hairs and the development of mechanical allodynia observed for both the ipsilateral (A) and contralateral (B) hindpaw. The timepoints are: baseline measured before first injection (BA), 3 h after first injection, and immediately before second injection (D-5), 3 h after the second injection, 24 h after the second injection (D-6), and on weeks 1–5 after the second injection (week). A significant increase in mechanical sensitivity occurred for both the ipsilateral and contralateral paws from 24 h after the second injection until 4 weeks (both paws). Data are presented as mean ± S.E.M., $^*P<0.05$ vs. baseline.

the exception of morphine, ([8-methyl-5-(4-(*N*,*N*-dimethylsulfamoyl)phenyl)-6,7,8,9,-tetrahydro-1H-pyrrolo[3,2-*h*]-iso-quinoline-2,3-dione-3-*O*-(4-hydroxybutyric acid-2-yl)oxime] NS1209 lamotrigine and mexiletine which have previously been tested using identical doses to those described in the current study (Erichsen and Blackburn-Munro, 2002). The animals were acclimatized to the revolving drum by a training run the day prior to drug testing. They were required to walk on the rotating rod, at 4 rpm for a maximum of 2 min. Only animals that showed no impairment in motor coordination (determined to be present if any rat fell more than two times during the recording period) were included for subsequent testing. On the following day, after baseline responses had been established, animals were administered appropriate drug treatments prior to further testing. Where applicable, the effective dose (ED_{50}) of drug required to induce ataxia (any animal falling more than two times) was calculated as the dose that induced motor impairments in 50% of the animals.

2.4. Drugs

Morphine hydrocloride was obtained from Mecobenzon (Denmark) and mexiletine hydrochloride was purchased from Sigma. NS1209 is a selective, competitive and potent AMPA receptor antagonist (Mathiesen et al., 1998) and was synthesised at NeuroSearch. Retigabine (*N*-(2-amino-4(4-fluorobenzylamino)-phenyl)carbamic acid ethyl ester) and flupirtine (Katadolon) were also synthesised at NeuroSearch A/S. Lamotrigine (Lamictal; 3,5-diamino-6-[2,3-dichlorophenyl]-1,2,4-triazine) was kindly supplied by GlaxoSmithKline (UK). Ketamine (Ketalar, 50 mg/ml), carprofen (Rimidryl Vet., 50 mg/ml) and diazepam were purchased from Unichem, Copenhagen, Denmark. Morphine, NS1209, mexiletine and diazepam were dissolved in physiological saline. Carprofen and ketamine were diluted from stock with physiological saline. Retigabine, flupirtine and lamotrigine were dissolved in Tween 80 and diluted to 10% with physiological saline. Morphine and carprofen were injected subcutaneously (s.c.), and all other drugs were administered intraperitonally (i.p.).

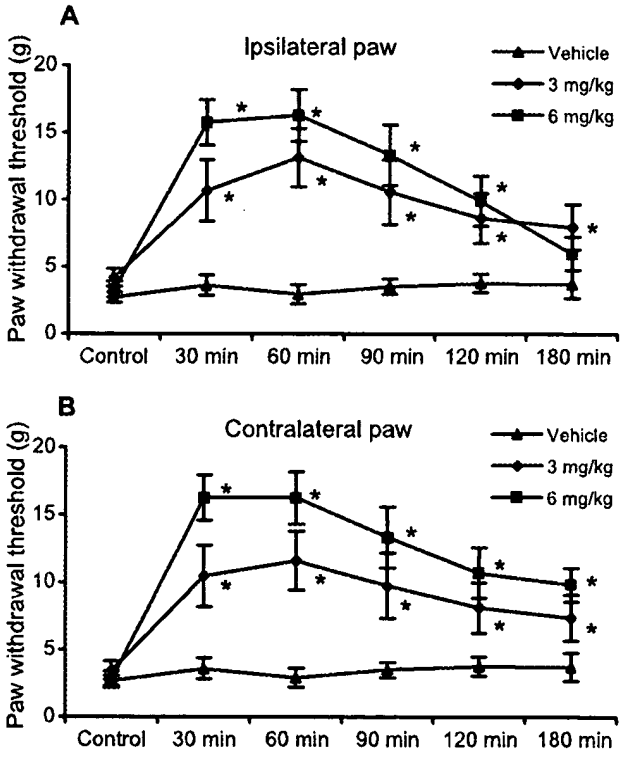

Fig. 2. Effects of morphine on hindpaw withdrawal threshold. Morphine (3 and 6 mg/kg, i.p.) was injected immediately after the baseline (control) response had been obtained. Morphine induced a dose-dependent increase in ipsilateral (A) and contralateral (B) paw withdrawal threshold, which remained significantly different from baseline and vehicle for up to 180 min after injection (ipsilateral paw for 120 min at morphine 6 mg/kg). Data are presented as mean ± S.E.M., $^*P<0.05$ vs. corresponding vehicle time points.

2.5. Statistics

Analysis of the data was performed using Statistica (version 6.0). Data for von Frey hair stimulation are presented as mean ± S.E.M. One way repeated analysis of variance (ANOVA) was used to analyse the overall effects of the treatments, followed by posthoc testing with Tukey's test to calculate the difference between groups. In all cases $P<0.05$ was considered to be statistically significant.

3. Results

3.1. General observations

From a total of 68 animals that received repeated injections of acidic saline, 49 (72%) showed a significant reduction in paw withdrawal threshold in response to von Frey hair stimulation 24 h after the second injection. The remaining 19 animals (28%) displayed reflex responses to innocuous mechanical stimulation that were similar to pre-injection levels and were excluded from the study. Twenty-four hours after the second injection, the withdrawal threshold to mechanical stimulation of the ipsilateral paw decreased from a pre-injection level of 16.2 ± 1.5 to 5.5 ± 0.9 g ($P<0.001$; Fig. 1). The contralateral paw showed a similar reduction in withdrawal threshold from a pre-injection level of 13.6 ± 1.6 to 4.6 ± 0.9 g ($P<0.001$). This increased sensitivity remained significant until 4 weeks after the second injection for both hindpaws. There was no significant difference in paw withdrawal threshold between the ipsilateral and contralateral sides. An additional eight animals, injected with saline at pH 7.4, showed no significant change in paw withdrawal threshold up to 5 weeks later (Fig. 1).

To test for the presence of mechanical hyperalgesia and cold allodynia both the ipsilateral and contralateral hindpaws were stimulated with a safety pin or ethyl chloride. No changes in paw withdrawal duration (all <0.5 s) or cold observation scores (0–1) were observed for either paw when compared with pre-surgery levels (all <0.5 s and 0–1, respectively), for up to 5 weeks after the second injection of acidic saline. Similarly, when tested for the presence of thermal hyperalgesia, there was no change in paw withdrawal latency to a noxious thermal stimulus for either the ipsilateral or contralateral sides at all time points when compared with pre-injection levels (7.4 ± 0.8 and 7.5 ± 0.4 s, respectively). Finally, in all experiments where drugs were tested, minimal significant differences in drug effects were observed between ipsilateral and contralateral

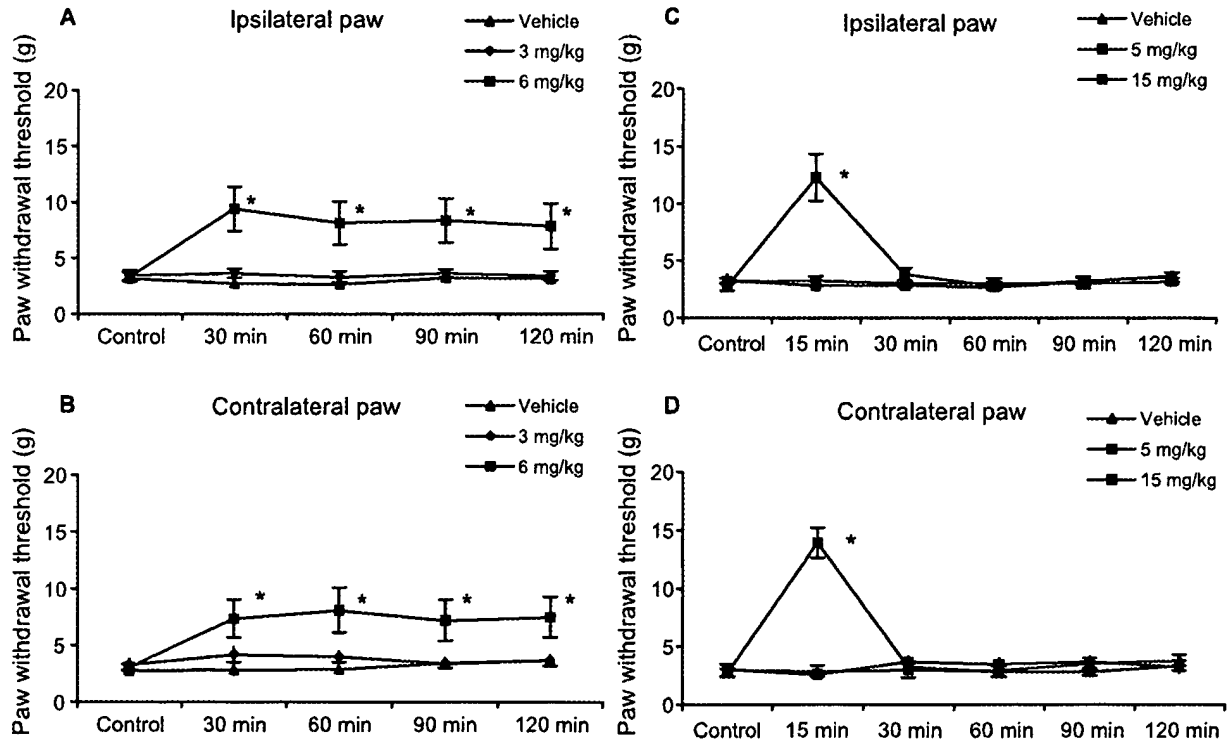

Fig. 3. Effects of glutamate receptor antagonists on hindpaw withdrawal threshold. (A, B) The AMPA receptor antagonist NS1209 (3 and 6 mg/kg, i.p.) was injected immediately after the baseline response (control) had been obtained. At the highest dose tested NS1209 increased paw withdrawal threshold from 30 min after injection and this remained significantly different from vehicle up to 120 min in both ipsilateral and contralateral paws. (C, D) The NMDA receptor antagonist ketamine (5 and 15 mg/kg, i.p.) was injected immediately after the baseline response had been obtained. Although ketamine significantly increased paw withdrawal threshold compared with vehicle (15 mg/kg), this effect was transient, lasting for only 15 min after injection for both hind paws. Data are presented as mean ± S.E.M., *$P<0.05$ vs. corresponding vehicle time points.

paw responses when compared to vehicle. Thus, in the following sections only values for the ipsilateral paw are given, except in those instances where differences between the two paws were observed.

3.2. Effects of morphine

Systemic administration of morphine produced a dose-dependent increase in paw withdrawal threshold in response to von Frey hair stimulation after repeated application of acidic saline injection (Fig. 2). The lowest dose of morphine (3 mg/kg, s.c.) significantly increased paw withdrawal threshold to 10.7 ± 2.2 ($P<0.0001$), compared with a threshold level of 3.4 ± 0.7 g, 30 min after injection of vehicle. The increase in paw withdrawal threshold remained significantly different from vehicle 180 min after injection (7.9 ± 1.6 g, $P<0.05$). At the highest dose of morphine tested (6 mg/kg, s.c.), the increase in paw withdrawal threshold was even greater 30 min post injection (15.7 ± 1.7 g, $P<0.0001$). This effect remained significantly different from vehicle up to 120 min for the ipsilateral paw (9.9 ± 1.9 g, $P<0.001$), and up to 180 min (9.8 ± 1.9 g, $P<0.005$) for the contralateral paw. We have previously shown that morphine has no effect on motor performance in the rotarod test when administered at the doses used in the current study (Erichsen and Blackburn-Munro, 2002).

3.3. Effects of glutamate receptor antagonists

3.3.1. NS1209

When the AMPA receptor antagonist NS1209 was administered i.p. at 3 mg/kg, no change in paw withdrawal threshold was observed when compared with injection of vehicle at any of the timepoints examined (Fig. 3A,B). In contrast, injection of the highest dose of NS1209 (6 mg/kg, i.p.) produced a significant increase in the paw withdrawal threshold to 9.4 ± 2.0 g ($P<0.001$) 30 min after injection when compared with vehicle-injected animals (2.8 ± 0.4 g). This elevation remained significant until 120 min after administration for the ipsilateral paw (7.8 ± 2.0 g, $P<0.05$) and for the contralateral paw (7.4 ± 1.7 g, $P<0.05$). We have previously shown that NS1209 has no effect on motor performance in the rotarod test when administered at the doses used in the current study (Erichsen and Blackburn-Munro, 2002).

3.3.2. Ketamine

Intraperitoneal injection of the NMDA receptor antagonist ketamine at 5 mg/kg, had no effect on paw withdrawal threshold when compared to vehicle for up to 120 min after administration (Fig. 3C,D). In contrast, the highest dose of ketamine (15 mg/kg, i.p.) produced a significant increase in paw withdrawal threshold to 12.2 ± 2 g ($P<0.0001$) from

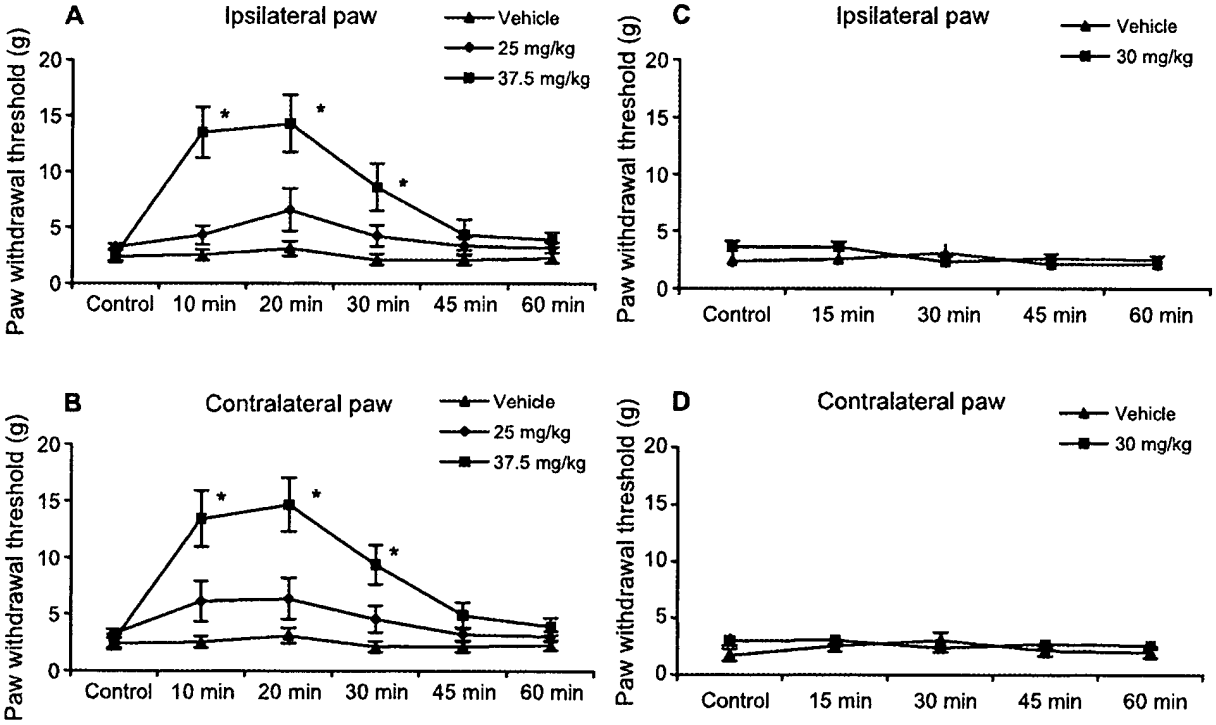

Fig. 4. Effects of voltage-activated Na^+ channel blockers on hindpaw withdrawal threshold. (A, B) The antiarrhythmic mexiletine (25 and 37.5 mg/kg, i.p.) was injected immediately after the baseline response (control) had been obtained. At the highest dose tested mexiletine induced a rapid increase in paw withdrawal threshold compared with vehicle. However, this effect was somewhat transient and had disappeared 45 min after injection. (C, D) Injection of the anticonvulsant lamotrigine (30 mg/kg, i.p.) immediately after the baseline response had been obtained had no effect on paw withdrawal threshold. Data are presented as mean ± S.E.M., $^*P<0.05$ vs. corresponding vehicle time points.

15 min after administration compared with injection of vehicle (2.8 ± 1.4 g). However, the increase was transient, and had returned to baseline levels 30 min after injection. At the highest dose of ketamine administered (15 mg/kg, i.p., $n=6$), no change in motor performance as assessed by the rotarod test was observed when compared with baseline (ED_{50}>15 mg/kg).

3.4. Effects of voltage-activated Na^+ channel blockers

3.4.1. Mexiletine

Administration of the anti-arrthymic mexiletine at the lowest dose of 25 mg/kg (i.p.) had no effect on paw withdrawal threshold when compared with injection of vehicle at any of the timepoints examined (Fig. 4A,B). However, when mexiletine was administered i.p. at a dose of 37.5 mg/kg paw withdrawal threshold was significantly increased to 13.5 ± 2.2 g ($P<0.0001$) within 10 min, when compared with injection of vehicle. This elevation in paw withdrawal threshold to von Frey hair stimulation remained significantly different from injection of vehicle until 30 min after drug administration (8.6 ± 2.1 g, $P<0.05$). We have previously shown that mexiletine has no effect on motor performance in the rotarod test when administered at the doses used in the current study (Erichsen and Blackburn-Munro, 2002).

3.4.2. Lamotrigine

Administration of the anticonvulsant lamotrigine (30 mg/kg, i.p.) had no effect on mechanical allodynia when compared with injection of vehicle at any of the timepoints examined (Fig. 4C,D). We have previously shown that lamotrigine has no effect on motor performance when administered at the same dose used in the current study (Blackburn-Munro et al., 2002).

3.5. Effects of KCNQ K^+ channel openers

3.5.1. Retigabine

When the KCNQ K^+ channel opener retigabine was administered at 3 mg/kg, (i.p.), no change in paw withdrawal threshold to von Frey hair stimulation was observed when compared with injection of vehicle at any time point (Fig. 5A,B). In contrast, injection of the highest dose of retigabine (10 mg/kg, i.p.) produced a significant increase ($P<0.0001$) in paw withdrawal threshold that reached a maximal level of 14.0 ± 1.5 g 30 min after injection when compared to vehicle administration (2.8 ± 0.3 g). This effect remained significantly different from vehicle for a further 30 min (6.6 ± 1.2 g, $P<0.01$). At the highest dose of retigabine tested, we observed that 50% of animals showed impaired motor performance 15 min after administration as assessed by the rotarod test when compared with baseline (ED_{50} = 10

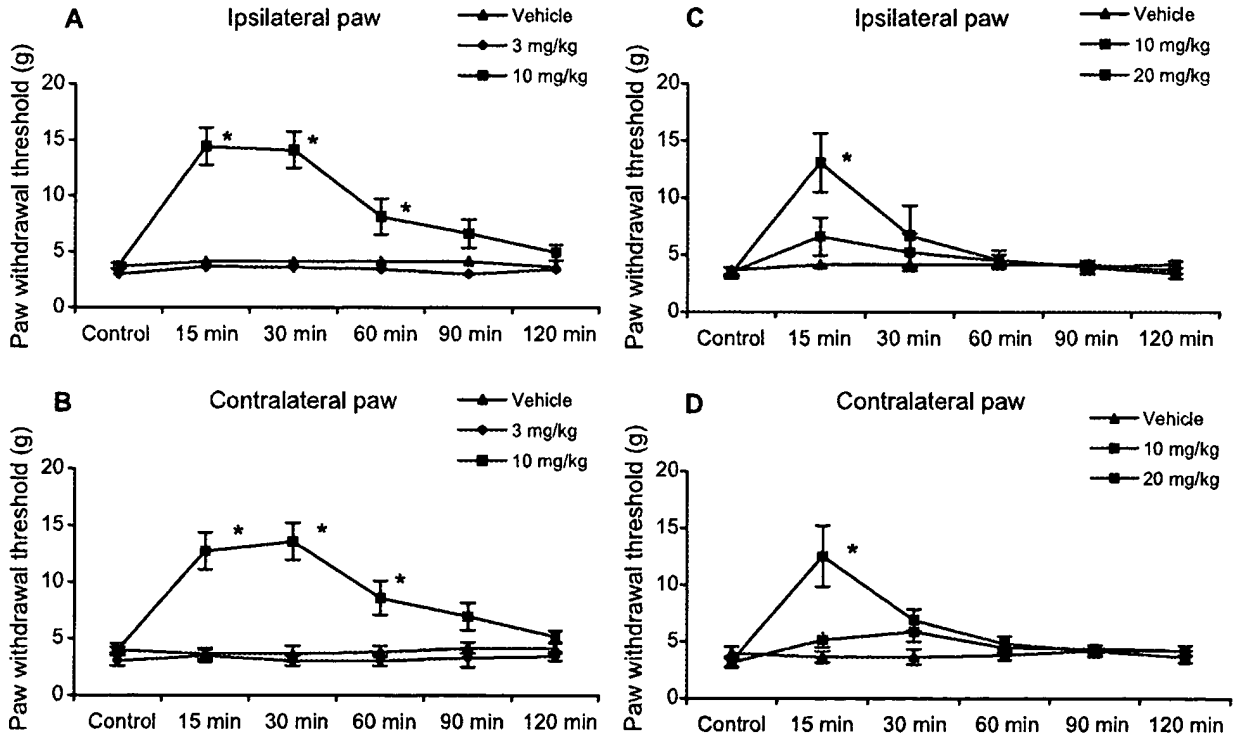

Fig. 5. Effects of KCNQ potassium channel openers on hindpaw withdrawal threshold. (A, B) Retigabine (3 and 10 mg/kg, i.p.) was injected immediately after the baseline response (control) had been obtained. At the highest dose tested retigabine increased paw withdrawal threshold from 15 min after injection and this remained significantly different from vehicle until 60 min after injection for both paws. (C, D) Flupirtine (10 and 20 mg/kg, i.p.) was injected after the baseline response had been obtained. At the highest dose tested a transient increase in paw withdrawal threshold was observed 15 min after administration for both paws. Data are presented as mean ± S.E.M., *$P<0.05$ vs. corresponding vehicle time points.

mg/kg). However, motor performance had returned to a baseline at after all time points (30, 45 and 60 min) examined after 15 min (ED_{50}>10 mg/kg).

3.5.2. Flupirtine

Intraperitoneal injection of flupirtine at 10 mg/kg, had no effect on paw withdrawal threshold in response to von Frey hair stimulation, when compared with vehicle injection for up to 120 min after administration (Fig. 5C,D). In contrast, 15 min after i.p. injection of 20 mg/kg flupirtine a significant increase in paw withdrawal threshold to 13.0 ± 2.6 g ($P<0.05$), was observed when compared to injection of vehicle (4.1 ± 0.3 g). However, this elevation in paw withdrawal threshold returned to a baseline level 15 min later, and remained at this level for the remainder of the experiment. At the highest dose of flupirtine administered (20 mg/kg, i.p., $n=6$), no change in motor performance was observed when compared with baseline (ED_{50}>20 mg/kg).

3.6. Effects of the cycloxygenase-2 inhibitor carprofen

Subcutaneous administration of the selective cycloxygenase-2 inhibitor carprofen at 10 and 15 mg/kg, had no effect on paw withdrawal threshold when compared to injection of vehicle at any of the timepoints examined up to 120 min post-injection (Fig. 6A). At the highest dose of carprofen administered (15 mg/kg, s.c., $n=6$), no change in motor performance was observed when compared with baseline (ED_{50}>15 mg/kg).

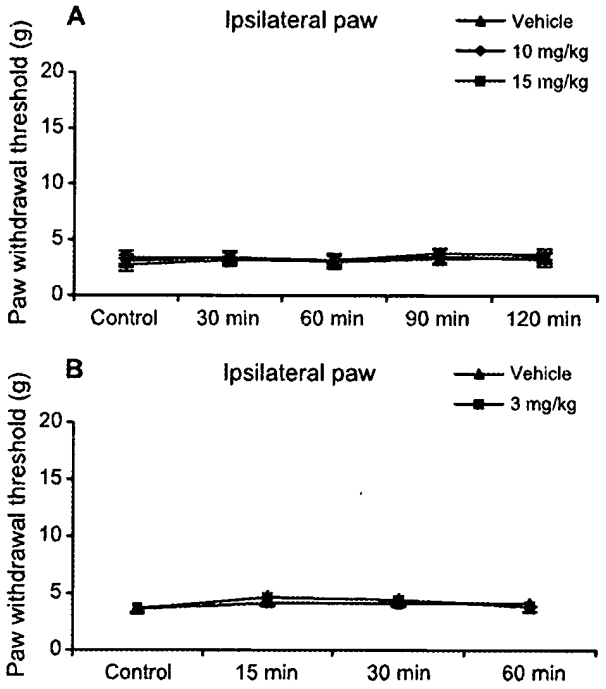

Fig. 6. Effects of carprofen and diazepam on hindpaw withdrawal threshold. (A) The cycloxygenase-2 inhibitor carprofen (10 and 15 mg/kg, i.p.) was injected immediately after the baseline response (control) had been obtained. Carprofen had no effect on paw withdrawal threshold, in the ipsilateral paw, at either dose tested. (B) The GABA modulator diazepam (3 mg/kg, i.p.) was injected immediately after the baseline response had been obtained. Diazepam had no effect on withdrawal threshold in response to von Frey hair stimulation of the ipsilateral hindpaw. Data are presented as mean ± S.E.M.

3.7. Effects of the gamma aminobutyric acid (GABA) modulator diazepam

Injection of the benzodiazepine GABA modulator diazepam at 3 mg/kg (i.p.) had no effect on paw withdrawal threshold when compared with injection of vehicle at any of the timepoints examined up to 60 min after administration (Fig. 6B). When tested subsequently in the rotarod test we observed that 16% of rats ($n=6$) displayed impaired motor performance 15 min after administration of diazepam (3 mg/kg). On this basis and in the light of the complete lack of antinociceptive effect for the 3 mg/kg dose we decided not to test higher doses in the current study.

4. Discussion

We have shown in the current study that repeated i.m. injection of acidic saline in the rat produces a long lasting bilateral hypersensitivity to innocuous mechanical stimulation of the hindpaw, which is in general agreement with previous observations made for this model (Sluka et al., 2001). We have then gone on to characterize this model pharmacologically by measuring hindpaw reflex nociceptive responses to von Frey stimulation after systemic administration of a range of drugs with proven analgesic efficacy in other animal models of chronic pain. We observed that morphine produced a dose-dependent and particularly prolonged reduction in mechanical hypersensitivity, whilst NS1209, ketamine, mexiletine, retigabine and flupirtine also attenuated mechanical hypersensitivity, although to a lesser extent than morphine. However, lamotrigine, carprofen and diazepam had no apparent effect on mechanical hypersensitivity under the conditions employed in the current study.

In their original description of this model Sluka and colleagues observed that the hindpaw sensitivity of rats ranged from 7 to 60 mN (approximately 0.7–6 g) after two unilateral injections of acidic saline (pH 4) administered 5 days apart; it was not reported if rats failed to develop hindpaw sensitivity within this range, thus suggesting that 100% of animals were 'responders' (Sluka et al., 2001). We used an identical injection protocol in the current study to observe an essentially similar decrease in paw withdrawal threshold to levels within the range of 0.94–5.41 g 24 h after the second injection of acidic saline. However, we excluded rats that did not show a post-injection withdrawal threshold below an upper level of 5.41 g (hence 72% of animals were regarded as responders), since we needed to leave an adequate window to test for marginal drug-induced reversal of hindpaw mechanical hypersensitivity.

Although increased sensitivity to mechanical stimuli, indicative of mechanical allodynia and hyperalgesia is well documented in fibromyalgia patients, altered thresholds to heat as well as cold-mediated sensory stimulation have also been documented (Price et al., 2002). Sluka et al. (2001) have previously reported that acid-induced chronic muscle pain in rats may more closely reflect the symptomologial profile of muscle pain in fibromyalgia patients, than other currently available animal models. Surprisingly however, these rats do not appear to show any changes in reflex responses to noxious, thermal stimulation of the hindpaw.

Subcutaneous administration of the selective cyclooxygenase-2 inhibitor carprofen at doses ranging from 5 to 15 mg/kg has previously been reported to attenuate a range of pain related behaviours over a 4–5 h period in a rat model of abdominal pain (Roughan and Flecknell, 2001). However, in the current study, we observed that carprofen had no effect of hindpaw mechanical hypersensitivity after administration, suggesting that ongoing peripheral inflammatory mediated events do not contribute to the pathophysiology of this model. A general lack of histological changes in the injected muscle after repeated i.m. acidic saline injection has been reported, suggesting that central sensitising events contribute to the aetiological development of acid-induced muscle pain (Sluka et al., 2001). This contrasts with carrageenan injection into the muscle, which also produces a long-lasting bilateral increase in hindpaw sensitivity, but is associated with marked inflammatory cell infiltration (Radhakrishnan et al., 2003), which might be expected to contribute to sensitisation of peripherally mediated sensory transmission. A similar lack of detectable peripheral pathology in fibromyalgia patients suggests that central pathophysiological processes also underlie the symptomological profile of the disease. Indeed, recent studies demonstrating that fibromyalgia patient's display enhanced wind-up to repetitive thermal and cold stimuli (Price et al., 2002), in conjunction with imaging studies showing pain-induced activation of several brain structures (Gracely et al., 2002) confirm such suggestions of altered central nociceptive processing. However, we were unable to detect any changes in other measures of pain-like behaviour in this model, which in addition to partially confirming previous observations of Sluka et al. (2001) reinforce the concept of a unique pathophysiology for fibromyalgic pain in humans.

4.1. Effects of morphine

The efficacy of opiate analgesics for the treatment of chronic pain in humans and alleviating pain-like behaviours in animal models, especially those of neuropathic origin, has been variably questioned (Arner and Meyerson, 1988). In spite of these observations we have previously shown that acute administration of morphine at doses comparable to those used in the current study effectively alleviate mechanical hyperalgesia and mechanical/cold allodynia in the spared nerve injury model of neuropathic pain (Erichsen and Blackburn-Munro, 2002). In the present study, administration of morphine produced a dose-dependent reversal in mechanical hypersensitivity in rats after repeated i.m. injection of acidic saline. This supports previous observations where spinal administration of μ-opioid receptor agonists has been shown to attenuate mechanical hypersensitivity in this model (Sluka et al., 2002). A particularly intriguing aspect of our findings pertaining to morphine was the unusually long duration of action of its anti-nociceptive effects which lasted for up to 3 h after administration. This long-lasting effect of morphine suggests that μ-opioid receptor levels located either pre- or postsynaptically to primary fibre input may be increased in this model, although other mechanisms such as persistent activation of downstream kinases (Gutstein et al., 1997) may also explain the prolonged duration of action.

4.2. Effects of glutamate receptor antagonists

AMPA receptor antagonists can prevent the development of hyperalgesia and allodynia observed in response to inflammatory stimuli, nerve and spinal cord injury (Fundytus, 2001; Simmons et al., 1998). We observed in the current study that the selective AMPA receptor antagonist NS1209 produced a robust increase in paw withdrawal threshold, lasting for 90 min in rats with acid-induced chronic pain. This essentially confirms the findings of a previous study where intrathecal administration of the AMPA receptor antagonist NBQX (1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[*f*]quinoxaline-7-sulfonamide disodium) also reduced the bilateral increase in mechanical hypersensitivity, although with a shorter duration of action than NS1209 (Skyba et al., 2002). NMDA receptor antagonists also reduce a wide spectrum of pain-like behaviours in animal models of chronic inflammatory and neuropathic pain (Fundytus, 2001). Intrathecal administration of the NMDA receptor antagonist D-[3H]2-amino-5-phosphonopentanoate (D-[3H]) AP5 has been shown to produce a robust bilateral attenuation of mechanical hypersensitivity in the model used in the current study, but at a dose associated with disturbance of motor function (20 nmol/10 μl) (Skyba et al., 2002); a less robust attenuation of mechanical hypersensitivity was observed at non-ataxic doses. In the present study, intraperitoneal administration of ketamine at a dose presumably devoid of motor impairing effects was associated with a marked, but transient increase in paw withdrawal threshold in response to von Frey hair stimulation. However, the lack of motor impairment observed at this dose is surprising based on the reports from other studies where moderate ataxia has been observed at similar dose levels (Danysz et al., 1994). This finding may relate to the rather low sensitivity of the constant speed rotarod used in this study. Similar results to those described above have been obtained in patients with fibromyalgia where intravenous infusion of ketamine

has been shown to reduce ongoing muscle pain and attenuate temporal summation of preconditioned electrical stimuli (Graven-Nielsen et al., 2000).

4.3. Effects of voltage-activated Na^+ channel blockers

Both mexiletine and lamotrigine have in common the ability to preferentially bind and stabilize inactivated states of the Na^+ channel to prevent Na^+ influx into cells (Clare et al., 2000; Graven-Nielsen et al., 2000). This property endows these drugs with the ability to selectively inhibit Na^+ channels during sustained depolarisation, such as that incurred in the setting of tissue injury. Administration of mexiletine (25–50 mg/kg, i.p.) produces a profound dose-dependent inhibition of second phase nociceptive behaviours in the rat formalin test (Blackburn-Munro et al., 2002). The ability of mexiletine within this dose range, to markedly attenuate hypersensitivity to mechanical and cold stimulation of the ipsilateral hindpaw, has also been recently described in two rat models of neuropathic pain (Erichsen et al., 2003). We also observed that mexiletine reduced hypersensitivity of the hindpaw in response to mechanical stimulation in rats after repeated i.m. injection of acidic saline, albeit with a relatively short duration of action. However, this duration of action is in keeping with the previously described anti-nociceptive effects reported for mexiletine. In contrast, administration of the anticonvulsant drug lamotrigine to rats in the current study had no effect on hindpaw hypersensitivity to mechanical stimulation. This was somewhat surprising given that numerous other studies have reported anti-nociceptive effects for lamotrigine in animal models of persistent and chronic pain (Blackburn-Munro et al., 2002; Erichsen et al., 2003; Nakamura-Craig and Follenfant, 1995). In particular administration of 15 mg/kg lamotrigine has been shown to markedly inhibit second phase pain behaviours in the formalin test (Blackburn-Munro et al., 2002). Furthermore in neuro-injured pain rat's mechanical hyperalgesia in response to pin prick stimulation is also attenuated by 10 mg/kg lamotrigine (Erichsen et al., 2003), while doses as high as 60 mg/kg have been shown to be ineffective in attenuating mechanical allodynia. Thus, we are reasonably confident that the dose of lamotrigine chosen in the present study was large enough to attenuate pain related behaviours. Whether pharmacokinetic differences in the way that mexiletine and lamotrigine bind to the channel (as has been shown for carbamazepine and phenytoin (Kuo et al., 1997) accounts for the disparity in affecting mechanical hypesensitvity in these models remains to be established.

4.4. Effects of KCNQ K^+ channel openers

The M-current is a subthreshold voltage-gated K^+ current that serves to stabilise the membrane potential and control neuronal excitability (Brown and Yu, 2000). Functional studies associate the M-current to homo- or hetero-multimers of KCNQ (2–5) protein subunits (Jentsch, 2000). The anticonvulsant drug retigabine, has been shown to activate KCNQ K^+ channels expressed in mammalian cells (Rundfeldt and Netzer, 2000) and native M-currents in rat sympathetic neurones (Tatulian et al., 2001; Wickenden et al., 2001).

Previously we have shown that oral administration of retigabine (20 mg/kg) attenuates second phase nociceptive behaviours in the rat formalin test (Blackburn-Munro et al., 2002). This attenuating effect of retigabine appeared to be selectively mediated by KCNQ K^+ channels, since it was completely reversed by prior administration of the KCNQ K^+ channel blocker (10,10-bis(4-pyridinylmethyl)-9(10H)-anthracenone) XE-991. In the same study retigabine also reduced mechanical hyperalgesic and cold allodynic responses of the ipsilateral hindpaw in the chronic constriction injury and spared nerve injury models of neuropathic pain. Interestingly, retigabine had no effect on mechanical allodynia as measured by paw withdrawal threshold in response to von Frey hair stimulation. In contrast, we have shown in the current study that i.p. administration of retigabine can reduce mechanical hypersensitivity to von Frey hair stimulation after repeated injection of acidic saline into the gastrocnemius muscle. The simplest explanation for this apparent discrepancy is that retigabine appears to have been administered at a maximally tolerated dose in the current study. We observed that 50% of animals had compromised motor performance as measured by the rotarod test 15 min after injection (although not at 30 and 60 min post-injection), whereas oral administration in the former study had no effect on motor performance at any time point measured. Recently, retigabine has been shown to interact with a site on the $GABA_A$ receptor complex raising the possibility that enhancement of GABA receptor function may contribute to its anticonvulsant properties (Van Rijn and Willems-van Bree, 2003). However, when we tested the $GABA_A$ receptor modulator diazepam in the current study, we saw no effect on hindpaw mechanical sensitivity, suggesting that retigabine does not mediate an anti-nociceptive effect in this model via such a mechanism of action.

Flupirtine is a centrally acting non-opioid analgesic, which is structurally similar to retigabine. Preliminary studies have shown that flurpirtine positively modulates transfected KCNQ K^+ channels with a similar mechanism of action to, but with less potency than retigabine (Ilyin et al., 2002). Other postulated mechanisms of action for the analgesic effects of flupirtine have included activation of inhibitory brainstem monoaminergic pathways which descend to the spinal dorsal horn (Szelenyi and Nickel, 1987; Szelenyi et al., 1989), to indirect inhibition of NMDA receptor activation via stabilization of the resting membrane potential (Kornhuber et al., 1999). Our results show that flupirtine is also less potent than retigabine in vivo, and that it also has a much shorter duration of antinociceptive action. Whilst we cannot definitively say that this action of flupiritine was mediated selectively via modulation of KCNQ K^+

channel function in view of the above observations, taken together with the results obtained for retigabine, it appears that KCNQ K^+ channels contribute to pain behaviour in this model of muscle pain.

In conclusion, we have confirmed previous observations that repeated application of i.m. acidic saline injection produces a long-lasting bilateral mechanical hypersensitivity to innocuous stimulation. This correlates with a number of symptoms presented in human chronic pain conditions, such as that observed in fibromyalgia patients where widespread muscoskeletal pain is regarded as a primary presenting symptom of the disease. However, in contrast to fibromyalgia patients we did not observe any mechanical hyperalgesia or hypersensitivity to thermal or cold stimuli applied to the hindpaw in this model. Overall, the present results suggest that in this model, muscle-mediated pain can be alleviated by various analgesics with differing mechanisms of action, and that once established ongoing inflammation does not appear to contribute to this process.

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Sodium Channel Modulators

Distinct types of sodium channels have been identified in the brain (Type I, Type II, Type III), heart (h_1) and skeletal muscle (u_1).

Compound	Pharmacological Action	References
Amiloride hydrochloride 3,5-Diamino-N-(aminoiminomethyl)-6-chloro-pyrazinecarboxamide M.W 266.11 Store at RT Soluble in water [2016-88-8]	**Cat. No. 0890** Na^+ channel blocker. Affinity for, or lack of, defines the I_{2A}-amiloride sensitive and I_{2B}-amiloride insensitive imidazoline binding sites.	Merck Index ***12*** 426. **Kleyman** *et al* (1988) Amiloride and its analogues as tools in the study of ion transport. J.Membr.Biol. ***105*** 1. **Ernsberger** *et al* (1992) A second generation of centrally acting antihypertensive agents act on putative I_1-imidazoline receptors. J.Cardiovasc.Pharmacol. ***20*** S1. **Hamill and McBride** (1996) The pharmacology of mechanogated membrane ion channels. Pharmacol.Rev. ***48*** 231.
Flecainide acetate *N*-(2-Piperidylmethyl)-2,5-*bis*-(2,2,2-trifluoroethoxy) benzamide acetate M.W. 474.40 Desiccate at +4°C Soluble to 50 mM in water [54143-56-5]	**Cat. No. 1470** Open Na^+ channel blocker that inhibits fast Na^+ current in cardiac muscle in a use- and concentration-dependent manner. Orally-active class Ic antiarrhythmic agent.	**Banitt** *et al* (1977) Antiarrhythmics. 2. Synthesis and antiarrhythmic activity of *N*-(piperidylalkyl) trifluoroethoxybenzamides. J.Med.Chem. ***20*** 821. **Singh** *et al* (1984) The electrophysiology and pharmacology of verapamil, flecainide, and amiodarone: correlations with clinical effects and antiarrhythmic actions. Ann.N.Y.Acad.Sci.USA ***17*** 251. **Rouet and Ducouret** (1994) Use- and concentration-dependent effects of flecainide in guinea pig right ventricular muscle. J.Cardiovasc.Pharmacol. ***24*** 177.
Flunarizine dihydrochloride (E)-1-[Bis(4-fluorophenyl)methyl]-4-(3-phenyl-2-propenyl)piperazine M.W. 477.43 Store at RT Soluble to 5 mM in ethanol [30484-77-6]	**Cat. No. 0522** Dual Na^+/Ca^{2+} channel blocker; a cerebral and peripheral vasodilator. Neuroprotective.	**Pauwels** *et al* (1991) Ca^{2+} and Na^+ channels involved in neuronal cell death - protection by flunarizine. Life.Sci. ***48*** 1881. **Eichler** *et al* (1994) The ability of diphenylpiperazines to prevent neuronal death in dorsal root ganglion neurons *in vitro* after axotomy. J.Neurochem. ***62*** 2148. **Urenjak and Obrenovitch** (1996) Pharmacological modulation of voltage gated Na^+ channels: a rational and effective strategy against ischemic brain damage. Pharmacol.Rev. ***48*** 21.
β-Pompilidotoxin β-PMTX M.W. 1557.90	**Cat. No. 1539** Novel wasp neurotoxin that slows Na^+ channel inactivation. Facilitates neuromuscular	**Konno** *et al* (1998) Isolation and structure of pompilidotoxins, novel peptide neurotoxins in solitary wasp venoms. Biochem.Biophys.Res.Commun. ***250***

Desiccate at -20°C Solubility: see Peptides [216064-36-7]	synaptic transmission and discriminates between rat neuronal and cardiac Na^+ channel α-subunits.	612. **Kinoshita** *et al* (2001) Novel wasp toxin discriminates between neuronal and cardiac sodium channels. Mol.Pharmacol. ***59*** 1457. **Miyawaki** *et al* (2002) Differential effects of novel wasp toxin on rat hippocampal interneurons. Neurosci.Lett. ***328*** 25.
QX 222 2-[(2,6-Dimethylphenyl)amino]-N,N,N-trimethyl-2-oxoethanaminium chloride M.W. 256.78 Store at RT Soluble to 100 mM in water [21236-55-5]	**Cat. No. 1043** Sodium channel blocker.	**Cuevas and Adams** (1994) Local anaesthetic blockade of neuronal nicotinic ACh receptor-channels in rat parasympathetic ganglion cells. Br.J.Pharmacol. ***11*** 663. **Hanck** *et al* (1994) Kinetic effects of quaternary lidocaine block of cardiac sodium channels: a gating current study. J.Gen.Physiol. ***103*** 19.
QX 314 N-(2,6-Dimethylphenyl carbamoylmethyl)triethylammonium bromide M.W. 343.31 Desiccate at +4°C Soluble to 100 mM in water [21306-56-9]	**Cat. No. 1014** Membrane impermeable quaternary derivative of lidocaine, a blocker of voltage-activated Na^+ channels.	**Stichartz** *et al* (1973) The inhibition of sodium currents in myelinated nerve by quaternary derivatives of lidocaine. J.Gen.Physiol. **Alreja and Aghajanian** (1994) QX-314 blocks the potassium but not the sodium dependent components of the opiate response in locus coeruleus neurons. Brain Res. ***639*** 320. **Perkins and Wong** (1995) Intracellular QX-314 blocks the hyperpolarization activated inward current Iq in hippocampal CA1 pyramidal cells. J.Neurophysiol. ***72*** 911.
Riluzole hydrochloride 2-Amino-6-trifluoromethoxy benzothiazole M.W. 270.66 Store at RT Soluble to 100 mM in DMSO [1744-22-5]	**Cat. No. 0768** Novel psychotropic agent with anticonvulsant, hypnotic, anxiolytic, anti-ischaemic and anaesthetic properties. Riluzole is able to act as a glutamate release inhibitor, blocks voltage dependent Na^+ channels and inhibits GABA uptake by striatal synaptosomes.	**Benazzouz** *et al* (1995) Riluzole prevents MPTP-induced parkinsonism in the rhesus monkey: a pilot study. Eur.J.Pharmacol. ***284*** 299. **Umeniya and Berger** (1995) Inhibition by riluzole of glycinergic postsynaptic currents in rat hypoglossal motoneurones. Br.J.Pharmacol. ***116*** 3227. **Taylor and Meldrum** (1995) Na+ channels as targets for neuroprotective drugs. TiPS. ***16*** 309. **Song** *et al* (1997) Differential action of riluzole on tetrodotoxin-sensitive and tetrodotoxin-resistant sodium channels. J.Pharmacol.Exp.Ther. ***282*** 707.
Tetrodotoxin Octahydro-12-(hydroxymethyl)-2-imino-5,9:7,10a-dimethano-10aH-[1,3]dioxocino[6,5-d] pyrimidine-4,7,10,11,12-pentol M.W. 319.27 Desiccate at +4°C Soluble in acidic buffer (pH 4.8) [4368-28-9]	**Cat. No. 1078** Selective inhibitor of Na^+ channel conductance. Binding is reversible and of high affinity (K_d = 1-10 nM). Blocks in a use-dependent manner.	Merck Index ***12*** 9382. **Kao** (1972) Pharmacology of tetrodotoxin and saxitoxin. Fed.Proc. ***31*** 1117. **Kao** (1986) Structure-activity relations of tetrodotoxin, saxitoxin, and analogues. Ann.N.Y.Acad.Sci. ***479*** 52. **Gleitz** *et al* (1996) The protective action of tetrodotoxin and (±)-kavain on anaerobic glycolysis, ATP content and intracellular Na^+ and Ca^{2+} of anoxic brain vesicles.

		Neuropharmacology ***35*** 1743.
Tetrodotoxin citrate Octahydro-12-(hydroxymethyl)-2-imino-5,9:7,10a-dimethano-10aH-[1,3]dioxocino[6,5-d] pyrimidine-4,7,10,11,12-pentol M.W. 511.40 Desiccate at +4°C Soluble in water [18660-81-6]	**Cat. No. 1069** Highly selective, reversible sodium channel blocker; citrate salt of tetrodotoxin.	Merck Index ***12*** 9382. **Kao** (1972) Pharmacology of tetrodotoxin and saxitoxin. Fed.Proc. ***31*** 1117. **Kao** (1986) Structure-activity relations of tetrodotoxin, saxitoxin, and analogues. Ann.N.Y.Acad.Sci. ***479*** *52*. **Gleitz** *et al* (1996) The protective action of tetrodotoxin and (±)-kavain on anaerobic glycolysis, ATP content and intracellular Na^+ and Ca^{2+} of anoxic brain vesicles. Neuropharmacology ***35*** 1743.
Vinpocetine (3α,16α)-Eburnamenine-14-carboxylic acid ethyl ester M.W. 350.46 Store at RT Soluble in DMSO and ethanol [42971-09-5]	**Cat. No. 0757** Phosphodiesterase inhibitor, selective for PDE1 (IC_{50} = 21 μM). Also blocks voltage-gated Na^+ channels.	Merck Index ***12*** 10128. **Moln? and Erd?**(1995) Vinpocetine is as potent as phenytoin to block voltage-gated Na^+ channels in rat cortical neurons. Eur.J.Pharmacol. ***273*** 303.

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Antinociceptive Effects of Sodium Channel-Blocking Agents on Acute Pain in Mice

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Abstract. The effects of various sodium channel blocking agents on acute thermal and mechanical nociception, as assessed using the plantar and tail pressure tests, respectively, were compared with the effects of morphine. The drugs used were mexiletine, lidocaine, carbamazepine, phenytoin, eperisone, tolperisone, and zonisamide. The sodium channel blocking agents exhibited a rather preferential elevation of the threshold for thermal nociception. By contrast, morphine produced similar analgesic effects on thermal and mechanical nociception. In the sciatic nerve isolated from mice, mexiletine, lidocaine, eperisone, and tolperisone impaired the propagation of low frequency action potentials (evoked at 0.2 Hz). Carbamazepine, phenytoin, and zonisamide generated a more frequency-dependent local anesthetic action with their obvious effects on higher frequency action potentials (evoked at 5 and/or 10 Hz). Our results show that sodium channel blocking agents have a preferential antinociceptive action against thermal stimulation that is likely to be attributed to their local anesthetic action.

Keywords: sodium channel blocker, plantar test, tail pressure test, local anesthetic action

Introduction

Some local anesthetics and antiepileptic agents are used clinically in the treatment of neuropathic pain (1 – 4). These drugs generally possess sodium channel blocking properties, and experiments involving animal models of neuropathic pain (rat) have in fact revealed that the sodium channel blocking agents exhibit analgesic effects (5 – 7). However, little is known about the role of sodium channels in the generation or conduction of acute or chronic pain signals. So far, there has been no study comparing the antinociceptive effects of different sodium channel blocking agents on acute pain.

In the study presented here, we investigated the effects of lidocaine, mexiletine, carbamazepine, phenytoin, zonisamide, eperisone, and tolperisone on the acute pain. Lidocaine and its structural analog mexiletine are class 1b antiarrhythmic drugs; carbamazepine, phenytoin, and zonisamide are used to treat epilepsy; eperisone and tolperisone are centrally acting muscle relaxants. Thus, although the principal therapeutic applications of these drugs are different, their common and major pharmacological target is thought to be sodium channels.

The goal of this study was to assess whether these sodium channel blocking agents generate qualitatively similar analgesic effects on the acute pain induced by thermal and mechanical stimulation.

Materials and Methods

All experimental protocols used were approved by the Animal Care and Use Committee of Nagoya City University and were conducted according to the guidelines of the National Institutes of Health and the Japanese Pharmacological Society.

Animals

Adult male ddY mice (5-week-old) were used in all experiments. Animals were allowed free access to food and water on a 12 h light/dark cycle in rooms where temperature and humidity were controlled.

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Effects on acute nociception

The degree of antinociception was determined using the plantar test and the tail pressure test.

Plantar test (nociceptive thermal stimulation): The degree of acute thermal nociception was assessed using the plantar test (Ugo Basile, Comerio, Italy) following a modification of the method of Hargreaves et al. (8). Mice were placed in a clear plastic chamber with a glass floor and allowed to acclimate to their environment before testing. During this time, mice initially demonstrated exploratory behavior, but subsequently stopped exploring and stood quietly with occasional bouts of grooming. A mobile radiant heat source, located under the glass floor, was focused onto the plantar surface of the left hindpaw, and paw withdrawal latencies (PWLs) were recorded. PWLs were measured twice for the left hindpaw of each animal, and the mean of the two values was used for calculations. The intensity of the heat stimulus was adjusted so that the baseline latency was 6 s and a 15-s cut-off time was imposed to avoid tissue damage.

Tail pressure test (nociceptive mechanical stimulation): Following the plantar test, mice were subjected to the tail pressure test (Pressure Analgesy-Meter; Muromachi Kikai, Tokyo) to assess their threshold for acute mechanical nociception. Pressure was applied about 1.5 cm from the base of the tail via a blunt probe. The pressure level was increased at a rate of 10 mmHg/s, and the pressure (mmHg) required to elicit a response was determined for each mouse; that pressure was defined as the nociceptive threshold. Tail pressure measurements were taken twice, and the mean of the two values was used for calculations. The cut-off pressure was 100 mmHg.

Local anesthetic action

The sciatic nerves were isolated from male ddY mice anesthetized with 80 mg/kg of intraperitoneally applied thiopental, and the desheathed peroneal nerve bundle was placed on the chamber in vitro. One end of the nerve was stimulated (0.2 – 10-Hz rectangular pulses, 0.05 ms in duration, 5 V), and action potentials were recorded from the other end of the nerve. Bipolar Ag-AgCl wire electrodes were used for stimulation and recording. Action potentials were displayed on an oscilloscope (Nihon Kohden, Tokyo), and eight consecutive responses were averaged by an averager (Nihon Kohden). Drugs were applied to the bath, which was filled with Locke-Ringer solution (see below, 21 – 24°C) and situated between the stimulating and recording electrodes. The concentration of drug in the bath was increased every 5 min by exchanging the drug solution (9).

Drugs

Mexiletine HCl was obtained from Sigma Chemical (St. Louis, MO, USA). Lidocaine HCl was obtained from Iwaki Seiyaku (Tokyo), carbamazepine and phenytoin from Wako Chemical (Tokyo), tolperisone HCl from Nippon Kayaku (Tokyo), eperisone HCl from Eisai (Tokyo), and morphine HCl from Shionogi (Osaka). Zonisamide was donated by Dainippon Pharmaceutical (Osaka). For measurements of the effects on acute pain, carbamazepine and phenytoin were suspended in 8 ml of propylene glycol and 2 ml of 3% Tween 80 solution (10), and the other drugs were dissolved in 0.9% w/v physiological saline. Drugs (or saline or propylene glycol and Tween 80 for control animals) were administered subcutaneously (s.c.) at 10 ml/kg.

For the measurements of local anesthetic properties, with the exception of carbamazepine and phenytoin, the drugs were dissolved in Locke-Ringer solution containing 154 mM NaCl, 5.6 mM KCl, 2.4 mM $NaHCO_3$, 1.6 mM $CaCl_2$, 1 mM $MgCl_2$, and 2.8 mM D-glucose, adjusted to pH 7.4 with HCl. Carbamazepine and phenytoin was dissolved in dimethyl sulfoxide (DMSO), and was then diluted to the final concentration (0.3 mM) with Locke-Ringer solution (0.1% DMSO).

Statistical analyses

All data are expressed as means ± S.E.M. The effects of drugs on the nociceptive threshold in both tests were evaluated in a time-course study, where each drug was administered at time zero. The nociceptive threshold of each time point was normalized to the pre-drug value. The dose-dependent analgesic actions of the drugs were assessed using the area under the time-course curve (AUC) between time zero and 45 min.

The amplitudes of action potentials were expressed as percentages of the corresponding values obtained before drug application.

One-way analysis of variance (ANOVA) followed by the two-tailed multiple *t*-test with Bonferroni correction (11) was used for multiple comparisons of control and treated groups. In the study of the local anesthetic action, the paired *t*-test was used to compare the action potential amplitudes between before and during drug application. Differences at $P<0.05$ were considered to be significant.

Results

The effects of test drugs on the nociceptive threshold were evaluated in a time-course study (Fig. 1 and 2) as well as using the area under the time-course curve (AUC, Fig. 3).

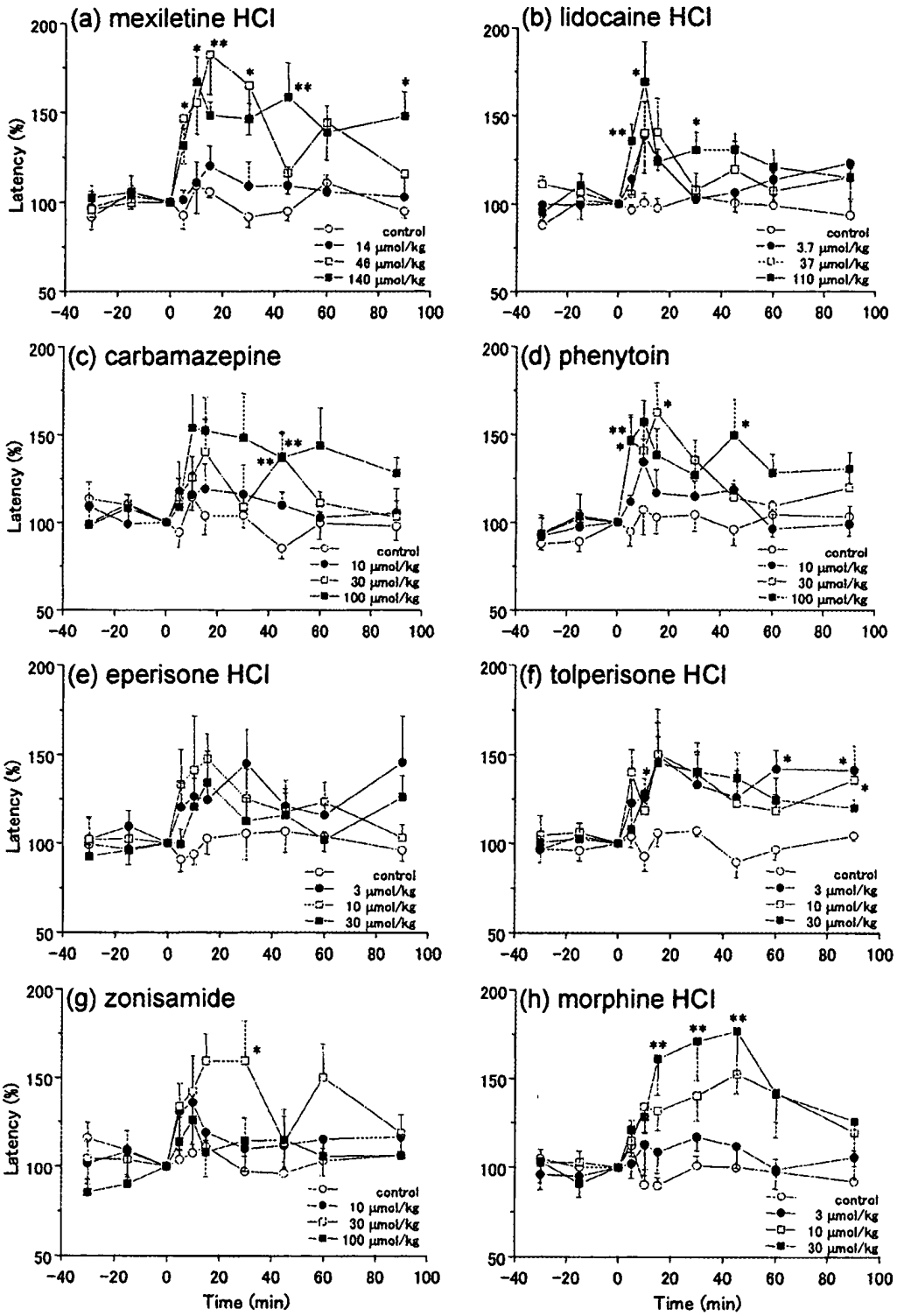

Fig. 1. The antinociceptive effects of sodium channel blocking agents on the paw-withdrawal (noxious heat-induced) threshold, as measured by the plantar test, in mice. a: mexiletine HCl (14, 46, and 140 μmol/kg, s.c.), b: lidocaine HCl (3.7, 37, and 110 μmol/kg, s.c.), c: carbamazepine (10, 30, and 100 μmol/kg, s.c.), d: phenytoin (10, 30, and 100 μmol/kg, s.c.), e: eperisone HCl (3, 10, and 30 μmol/kg, s.c.), f: tolperisone HCl (3, 10, and 30 μmol/kg, s.c.), g: zonisamide (10, 30, and 100 μmol/kg, s.c.), and h: morphine HCl (3, 10, and 30 μmol/kg, s.c.). Each data point represents the mean ± S.E.M. of six mice per group. Ordinates: mean latency expressed as a percentage of the corresponding value at time 0. Abscissae: time in minutes after drug administration. The significance of differences between the test and control values was determined by analysis of variance (ANOVA) followed by the two-tailed multiple *t*-test with Bonferroni correction (3 comparisons in 4 groups). $^{*}P<0.05$ and $^{**}P<0.01$ *vs* control in respective time.

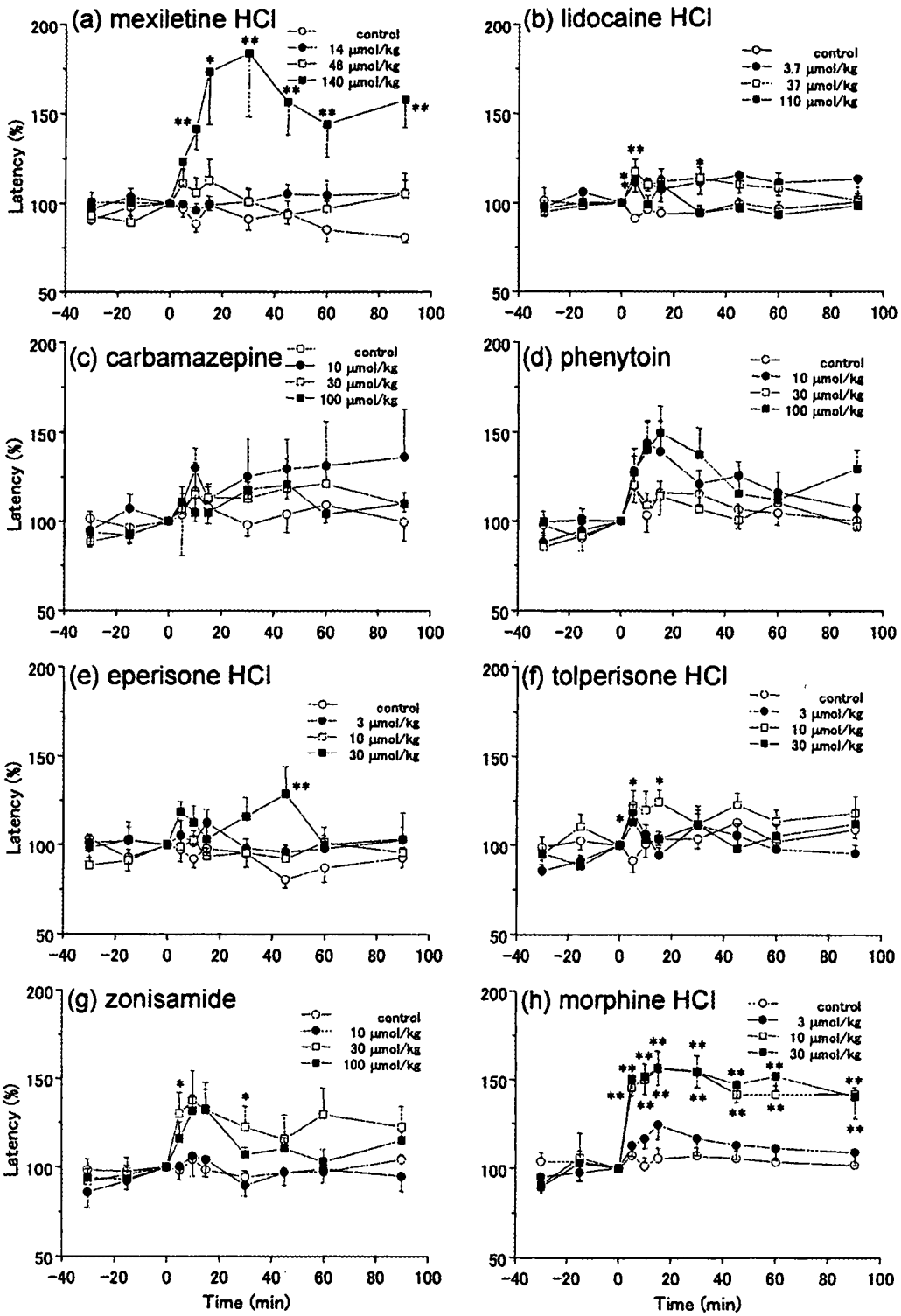

Fig. 2. The antinociceptive effects of sodium channel blocking agents on the mechanically induced pain threshold, as measured by the tail pressure test, in mice. a: mexiletine HCl (14, 46, and 140 μmol/kg, s.c.), b: lidocaine HCl (3.7, 37, and 110 μmol/kg, s.c.), c: carbamazepine (10, 30, and 100 μmol/kg, s.c.), d: phenytoin (10, 30, and 100 μmol/kg, s.c.), e: eperisone HCl (3, 10, and 30 μmol/kg, s.c.), f: tolperisone HCl (3, 10, and 30 μmol/kg, s.c.), g: zonisamide (10, 30, and 100 μmol/kg, s.c.), and h: morphine HCl (3, 10, and 30 μmol/kg, s.c.). Each data point represents the mean ± S.E.M. of six mice per group. Ordinates: mean latency expressed as a percentage of the corresponding value at time 0. Abscissae: time in minutes after drug administration. The significance of differences between the test and control values was determined by ANOVA followed by the two-tailed multiple *t*-test with Bonferroni correction (3 comparisons in 4 groups). $^{*}P<0.05$ and $^{**}P<0.01$ *vs* control at the respective time.

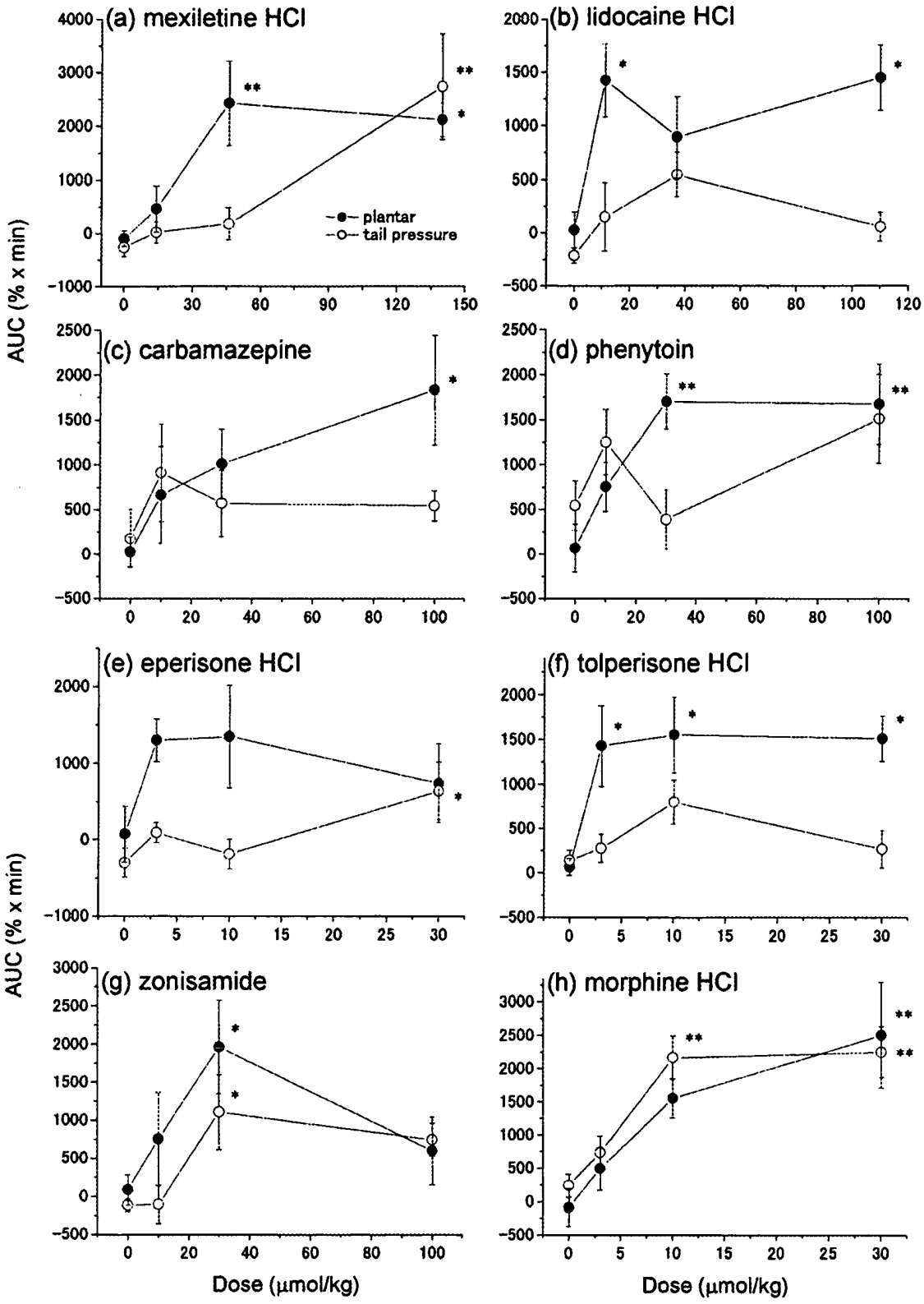

Fig. 3. Dose-response relationships of the analgesic effects of the sodium channel blockers on thermal and mechanical nociception. The effects of drugs were evaluated on the basis of the area under the time-course curve (AUC) between time zero (drug administration) and 45 min. Each value represents the mean ± S.E.M. of six mice. Ordinate: mean AUC (% × min) for the plantar and tail pressure tests (closed and open circle, respectively). The significance of differences between the test and control values was determined by ANOVA followed by the two-tailed multiple *t*-test with Bonferroni correction (3 comparisons in 4 groups). **P*<0.05 and ***P*<0.01 *vs* control.

Effects on the plantar test (nociceptive thermal stimulation)

Mexiletine HCl (14 – 140 μmol/kg equivalent to 3 – 30 mg/kg), carbamazepine (10 – 100 μmol/kg = 2.4 – 24 mg/kg), and phenytoin (10 – 100 μmol/kg = 2.5 – 25 mg/kg) elevated the withdrawal threshold for the plantar test in a dose-dependent manner (Fig. 1: a, c, and d; Fig. 3: a, c, and d). A similar elevation of the withdrawal threshold was obtained with lidocaine HCl (3.7 – 110 μmol/kg = 1 – 30 mg/kg, Fig. 1b and Fig. 3b); eperisone HCl (3 – 30 μmol/kg = 0.9 – 9 mg /kg, Fig. 1e and Fig. 3e, statistically not significant); tolperisone HCl (3 – 30 μmol/kg = 0.85 – 8.5 mg/kg, Fig. 1f and Fig. 3f); and zonisamide (3 – 100 μmol/kg = 0.6 – 21 mg/kg, Fig. 1g and Fig. 3g), while zonisamide generated antinociception with a bell-shaped dose-response relation (Fig. 3). The maximal effect of these drugs on the withdrawal threshold corresponded to that observed with 10 – 30 μmol/kg morphine HCl (= 3.2 – 9.7 mg/kg Fig. 1h and Fig. 3h). The muscle relaxant effects of eperisone and tolperisone are not thought to contribute to the elevation of the withdrawal threshold observed at the dose ranges used here (12). Thus, the sodium channel blockers examined in this study exhibit analgesic effects, as assessed using acute nociceptive thermal stimulation.

Effects on the tail pressure test (nociceptive mechanical stimulation)

Mexiletine HCl (140 μmol/kg) and zonisamide (30 – 100 μmol/kg) elevated the nociceptive threshold for the tail pressure test. The maximal effects of mexiletine HCl and zonisamide corresponded to those of 10 – 30 and 3 – 10 μmol/kg of morphine HCl, respectively (Fig. 2: a, g, and h; Fig. 3: a, g, and h). However, unlike the marked antinociception against thermal stimulation, the sodium channel blockers tested here generally demonstrated weak analgesic effects against mechanical stimulation.

Local anesthetic action

A summary graph of the local anesthetic action of sodium channel blockers tested in this study is shown in Fig. 4. When the action potentials were evoked at 0.2 Hz, a concentration-dependent inhibition of action potential propagation was obtained with mexiletine, lidocaine, eperisone, and tolperisone (Fig. 4A). Carbamazepine (0.3 mM), phenytoin (0.3 mM), and zonisamide (up to 3 mM) demonstrated no or little impairment of action potential propagation evoked at this low frequency. They required rather short intervals between propagated action potentials (5 and/or 10 Hz) to generate the apparent local anesthetic action (Fig. 4B).

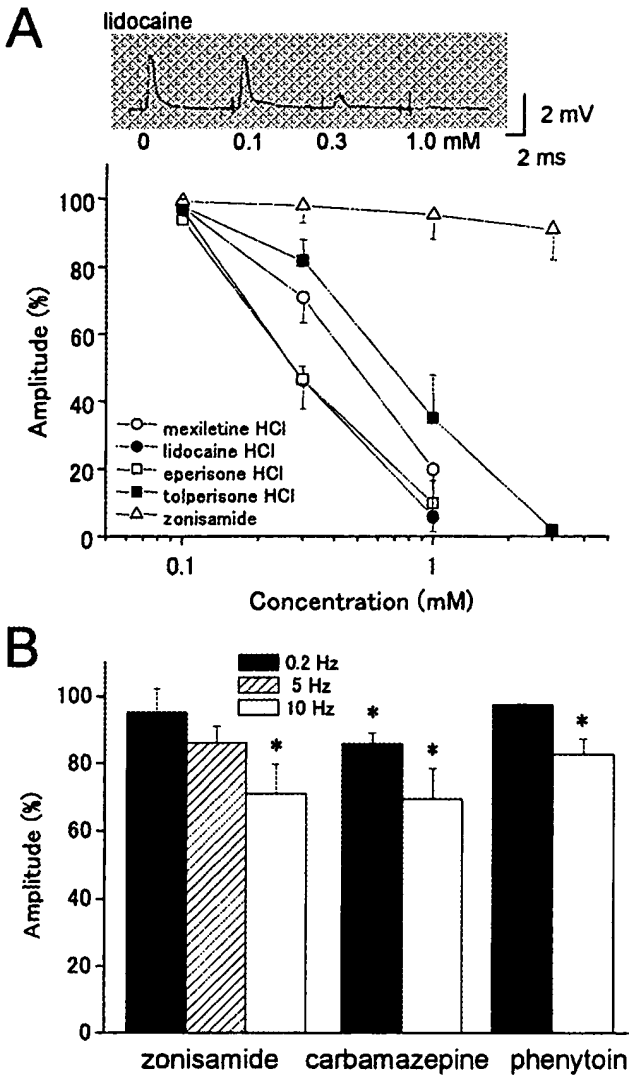

Fig. 4. Concentration-response relationships and frequency-dependency of the blocking effects of the sodium channel blockers on conductivity in the isolated mouse sciatic nerve. Each data point represents the mean ± S.E.M. of three to four preparations taken from different mice for each group. A: concentration-dependency. Action potentials were evoked at 0.2 Hz. Ordinate: mean amplitude of action potentials expressed as a percentage of the corresponding value before drug application. Abscissa: concentration of drugs. Upper traces show a representative effect of lidocaine HCl on the propagation of action potentials. B: frequency-dependency (0.2, 5, and/or 10 Hz) in the local anesthetic action of zonisamide (1 mM), carbamazepine (0.3 mM), and phenytoin (0.3 mM). *$P<0.05$ *vs* predrug control.

Without these drugs, the propagated action potentials were stably recorded when evoked at 0.2, 5, or 10 Hz.

Discussion

We have compared the antinociceptive effects of various sodium channel blocking agents on acute

thermal and mechanical pain and have demonstrated that these agents have a preferential antinociceptive action against thermal stimulation. These agents generated the local anesthetic action that may play a role in the differentiation between thermal and mechanical nociception.

There exist various distinctions in the mechanisms mediating the generation and conduction of thermal and mechanical pain signals. In the conduction of nociceptive pain signals to the spinal dorsal horn neurons, Doucette et al. (13) reported that C-fibers are activated in response to noxious heat stimuli. Moreover, Yeomans et al. (14) demonstrated that the types of nociceptors (C- and/or Aδ-fibers) activated by thermal nociception depend on the rates of skin heating; low rates of heating preferentially activates C-fiber nociceptors, while high rates of skin heating preferentially activate Aδ nociceptors. By contrast, both Aδ- and C-fibers are activated in response to noxious mechanical stimuli (15). In addition, distinct receptors mediate distinct nociceptive signals. Acid-sensing ion channels are related to mechanical nociception (16), and capsaicin receptors mediate heat nociception (17). Furthermore, thermal and mechanical nociception are modulated differentially by the descending monoaminergic pathways at the spinal level. The absence of norepinephrine in the central nervous system results in thermal hyperalgesia (18). Thus, various factors contribute to the differences between the conduction and modulation of thermal and mechanical nociception. Our observation that thermal nociception was more sensitive to sodium channel blocking agents discloses a new pharmacological characteristic.

Sodium channel blocking agents block the propagation of action potentials, which in the present study may have taken place at either Aδ- or C-fibers conducting pain signals to the spinal dorsal horn neurons. The drugs used here exhibited local anesthetic actions on the isolated sections of sciatic nerve. Most of the agents used here suppressed the propagation of low frequency action potentials (0.2 Hz). By contrast, to achieve the local anesthetic action by carbamazepine, phenytoin, and zonisamide required short intervals between action potentials. Such frequency-dependency is in agreement with the studies demonstrating that those compounds specially block the sustained repetitive firing of action potentials without altering the initial firing (19 – 21). Moreover, zonisamide enhances slow inactivation of sodium channels (22), as is also described for local anesthetics (23). Since small diameter neurons are generally more susceptible to the action of local anesthetics (24), it is conceivable that the local anesthetic action of sodium channel blocking agents used here contributes to their preferential analgesic action against C-fiber-mediated thermal nociception (13, 14), leaving Aδ- and C-fiber-mediated mechanical nociception (15) little affected. However, it remains unclear whether the analgesic effects of those drugs were derived solely from the blockade of peripheral sodium channels of Aδ- or C-fibers. Further studies are needed to determine the precise sites of action, including the central nervous system.

We consider that factors other than the local anesthetic action could contribute to a bell-shaped dose-response relation observed in the analgesic effect of zonisamide against thermal nociception because other sodium channel blocking agents here generally exhibited antinociception against thermal stimulation in a dose-dependent manner. Without a bell-shaped dose-response relation, zonisamide could demonstrated much evident preferential antinociception against thermal stimulation. This also requires further studies.

In conclusion, various drugs with sodium channel blocking actions preferentially suppressed thermal nociception, as contrasted with the equal effects of morphine on both thermal and mechanical nociception. This may be partly explained by the local anesthetic action of sodium channel blocking agents and differential sensitivities to local anesthetics of the fibers activated by thermal and mechanical nociception.

Acknowledgments

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Flupirtine shows functional NMDA receptor antagonism by enhancing Mg^{2+} block via activation of voltage independent potassium channels

Rapid Communication

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Summary. The spectrum of action of flupirtine includes analgesia, muscle relaxation and neuroprotection. N-methyl-D-aspartate (NMDA) receptor antagonism has been discussed as a possible mechanism of action of this compound with little direct evidence. The objective of the present study was to develop a plausible model to explain flupirtine's spectrum of action. A four-stage strategy was selected for this purpose: Firstly, the serum concentration of flupirtine under therapeutic conditions was determined on the basis of the current literature. The second stage involved assessing the known in-vitro effects in light of the therapeutic active concentration. Using whole cell patch clamp recordings from cultured rat superior colliculus neurones interactions between flupirtine and NMDA receptors were assessed. Only very high concentrations of flupirtine antagonized inward currents to NMDA (200 μM) at −70 mV with an IC_{50} against steady-state responses of 182.1 ± 12.1 μM. The effects of flupirtine were voltage-independent and not associated with receptor desensitization making actions within the NMDA receptor channel or at the glycine modulatory site unlikely. NMDA receptor antagonism probably has little relevance for the clinical efficacy of flupirtine as the concentrations needed were far higher than those achieved in clinical practice. However, the activation of a G-protein-regulated inwardly rectifying K^+ channel was identified as an interesting molecular target site of flupirtine. In the next stage, the central nervous spectrum of action of experimental K^+ channel openers (PCO) was considered. As far as they have been studied, experimental K^+ channel openers display a spectrum of action comparable to that of flupirtine. In the final stage, a global model was developed in which flupirtine stabilizes the resting membrane potential by activating inwardly rectifying K^+ channels,

thus indirectly inhibiting the activation of NMDA receptors. The model presented here reconciles the known functional NMDA receptor antagonism of flupirtine with the activation of K^+ channels that occurs at therapeutic concentrations, thus providing an understanding of flupirtine's spectrum of action. This makes flupirtine the prototype of a clinically applicable substance group with analgesic, muscle-relaxant and neuroprotective properties.

Keywords: Potassium channel, inwardly rectifying potassium channel, GIRK, flupirtine, N-methyl-D-aspartate receptor antagonist, patch clamp, superior colliculus culture.

Flupirtine (ethyl-N-[2-amino-6-(4-fluorophenylmethylamino)pyridin-3-yl] carbamate) has been in clinical use for many years as a centrally active analgesic with muscle-relaxant properties (Friedel and Fitton, 1993). In preclinical and preliminary clinical studies, neuroprotective (Schuster et al., 1999), antiepileptic (Sheridan et al., 1986) and antiparkinsonian (Schwarz et al., 1996) effects were additionally found. The mechanism of action of flupirtine has not been clear up to now. Although flupirtine does not have relevant affinity for any known recognition site on the NMDA receptor complex in binding studies (Osborne et al., 1996, 1998), antagonism of this receptor has recently been discussed at length as a possible mechanism of action of this compound (Osborne et al., 1994; Perovic et al., 1994; Schwarz et al., 1994, 1995; Timmann et al., 1995). This assumption is the expression of a misleading tendency to propose a precise mechanism of action at the receptor level purely on the basis of results from behavioral studies or neurotoxicity studies in vitro with inadequate pharmacological characterization of the interactions observed. Such speculations obviously neglect the complexity of neuronal networks, interactions between neuronal systems and the importance of processes up- and downstream of receptor activation in mediating end point parameters such as cell death. In view of this it seemed pertinent to test for possible interactions between flupirtine and NMDA receptors using whole cell patch clamp recordings from cultured neurones. This approach is better suited for the detection of direct antagonists or agonists at any site on the NMDA receptor and thus circumvents the need to propose yet another modulatory site if no interaction is observed.

Patch clamp recordings from cultured superior colliculus neurones were performed as described previously (Parsons et al., 1993a). In brief, superior colliculi were isolated from embryonic rats (E20–21) and maintained in culture for 11–14 days in $NaHCO_3$/HEPES-buffered minimum essential medium supplemented with 5% foetal calf serum and 5% horse serum (Gibco) and incubated at 37°C with 5% CO_2 (95% humidity). The superior colliculus culture was chosen for these experiments as it provides very stable recording conditions which are an absolute prerequisite for voltage-dependency and kinetic experiments. Moreover, the relatively small neurones (soma 15–20 μm Ø) are ideally suited to minimise problems of buffered diffusion for concentration clamp experiments. Finally, our own unpublished data indicate that

the somatic NMDA receptors expressed in cultured hippocampal and cortical neurones are similar.

Patch clamp recordings were made from these neurones with polished glass electrodes (4–5 MΩ) in the whole cell mode at room temperature (20–22°C) with the aid of an EPC-7 amplifier (List). Test substances were applied by switching channels of a custom made fast superfusion system with a common outflow (<10 ms exchange times). The contents of the intracellular solution were as follows (mM): CsCl (120), TEACl (20), EGTA (10), $MgCl_2$ (1), $CaCl_2$ (0.2), Glucose (10), ATP (2), cAMP (0.25); pH was adjusted to 7.3 with CsOH or HCl. The extracellular solutions had the following basic composition (mM): NaCl (140), KCl (3), $CaCl_2$ (0.2), glucose (10), HEPES (10), sucrose (4.5), tetrodotoxin (0.3 μM), glycine (1 μM), (pH 7.3). Flupirtine maleate was a generous gift of G. Pergande, ASTA Medica, Frankfurt. All other compounds were obtained from Sigma.

Very high concentrations of flupirtine antagonized inward current responses to NMDA (200 μM) at −70 mV (Fig. 1A) with an IC_{50} against steady-state responses of 182.1 ± 12.1 μM (Fig. 1B). Flupirtine did not enhance glycine-dependent desensitization in the continuous presence of the non-saturating concentration of glycine (1 μM), as evidence by the similar potency against the peak component of NMDA-induced currents (IC_{50} = 228.6 ± 8.9 μM). This probably excludes antagonistic actions at the strychnine-insensitive glycine modulatory ($glycine_B$) site of the NMDA receptor complex (Mayer et al., 1989; Parsons et al., 1993b) as previous observations indicate that most moderate to low affinity full $glycine_B$ antagonists are three to ten times more potent against steady-state than against peak currents. The effects of flupirtine (300 μM) were not voltage-dependent (Fig. 1C) and were apparently not use-dependent making channel blockade an unlikely candidate as the mechanism of NMDA receptor antagonism (Parsons et al., 1995). The Hill coeff. for NMDA receptor antagonism was close to unity and does not give any indication for cooperativity. Flupirtine alone did not evoke any measurable inward or outward current which also excludes direct agonistic actions at inhibitory $GABA_A$ or classical $glycine_A$ receptors as a possible mechanism of action as both of these receptors are expressed in these cultures and can be activated by their respective agonists under the conditions used (Parsons et al., 1993a).

The further intention of the present contribution was to provide an understanding of the mechanism of action. A four-stage strategy was selected to this end. The first stage was to determine the therapeutic serum concentration on the basis of the published data. In a second stage, the in-vitro effects of flupirtine were assessed to determine whether they are relevant under therapeutic conditions. In the next stage, flupirtine's spectrum of action was compared with that of experimental K^+ channel openers. Finally, a global model was developed to explain the clinical spectrum of action. In this contribution, we show that the spectrum of action of flupirtine can be understood within the context of the opening of inwardly rectifying K^+ channels. Flupirtine is thus the prototype of a new clinically applicable substance group with analgesic, muscle-relaxant and neuroprotective properties.

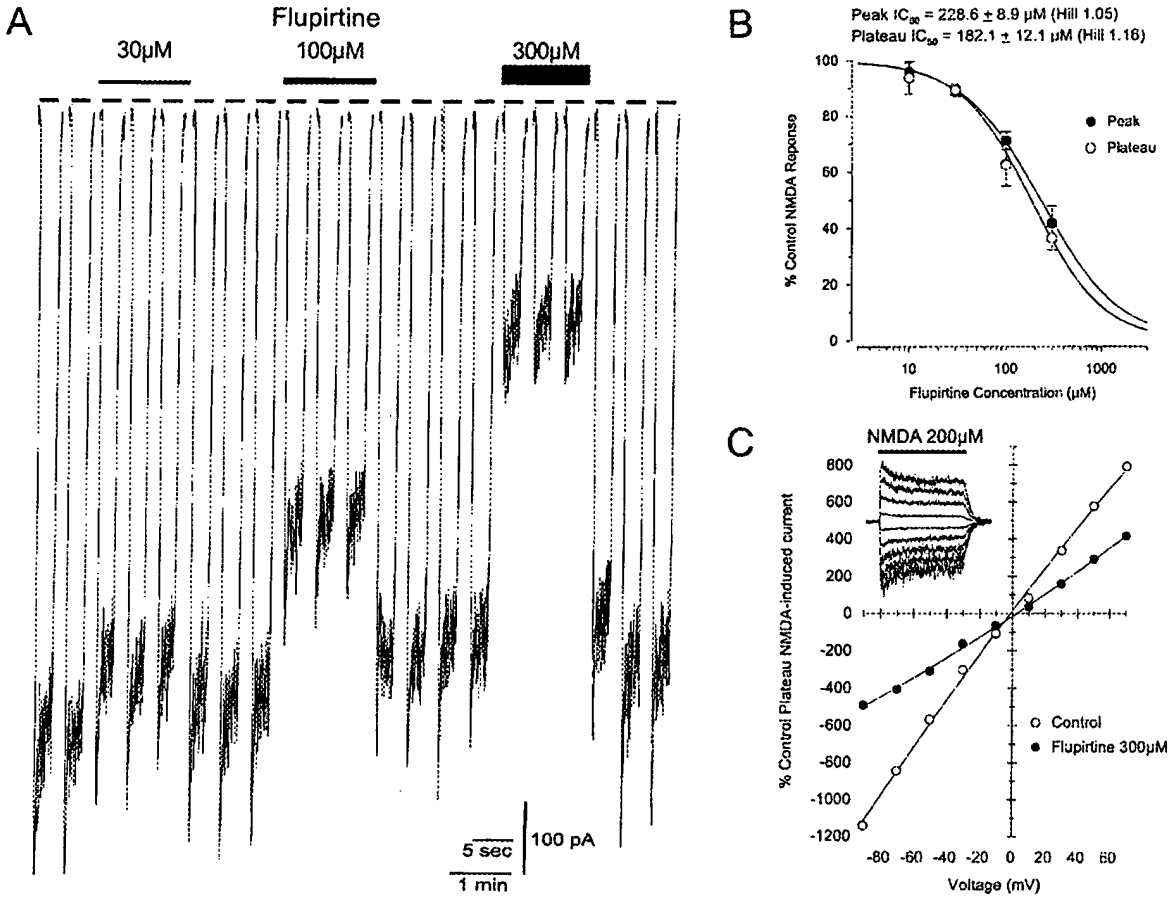

Fig. 1. **A** Concentration-dependence of the blockade of NMDA receptors by flupirtine on a single superior colliculus neurone. NMDA (200μM) was applied for 2.5 seconds every 30 seconds in the continuous presence of glycine (1μM) and at a constant membrane potential of −70mV. The inter-response interval has been omitted to allow better resolution of the kinetics of individual responses (blank spaces in trace). Flupirtine (30, 100 and 300μM) was continuously present for 1.5 minutes as indicated by the bars. **B** Concentration-dependence of the blockade of NMDA receptors by flupirtine. Peak and plateau (steady state) NMDA current response were normalised to control levels and plotted as means (±SEM) against flupirtine concentration (10μM n = 3, 30μM n = 7, 100μM n = 7, 300μM n = 5). Estimation of IC_{50}s and curve fitting were made according to the 4 parameter logistic equation (Grafit, Erithacus Software). **C** Voltage-independence of the blockade of NMDA receptors by flupirtine. NMDA (200μM) was applied for 2.5 seconds every 30 seconds in the continuous presence of glycine (1μM) at various membrane potentials. Plateau NMDA current responses in the absence and presence of flupirtine (300μM) have been plotted as means against membrane potential (n = 2). The upper left insert shows original data for the i.v. curve in the presence of flupirtine (300μM)

Therapeutically relevant concentrations of flupirtine

The therapeutically relevant concentrations of flupirtine are of decisive importance for assessing its molecular mechanisms of action. Of the various experimentally demonstrated effects of flupirtine (Table 1), only those that occur within a concentration range achieved under therapeutic conditions are relevant. Animal experiments show higher values in the brain than in the plasma 15 min after intravenous administration. After oral administration, comparable concentrations in the plasma and brain are measured within 30 min (Obermeier et al., 1985). Under therapeutic conditions, the plasma concentration is up to 6.5 μM; 2.5 μM are still detectable 12 h after the last intake (Hlavica and Niebch, 1985). The elimination half-life of

Table 1. A selection of the in-vitro effects of flupirtine

Effect investigated	Assay	Potency (μM)	Reference
NMDA channel	[^{3}H]MK-801 binding, cortex	>10	*
NMDA channel	Patch clamp, hippocampus	≫1	results presented here and Jakob and Krieglstein, 1997
α_1 receptor	[^{3}H]Prazosin binding, brain	>10	Szelenyi et al., 1989
α_2 receptor	[^{3}H]Clonidine binding, brain	>10	Szelenyi et al., 1989
5HT$_1$ receptor	[^{3}H]5-HT binding, frontal cortex	>100	Szelenyi et al., 1989
5HT$_2$ receptor	[^{3}H]Mesulergine binding, frontal cortex	75	Szelenyi et al., 1989
μ-opiate receptor	[^{3}H]Oxymorphone binding	>10	Nickel, 1987
δ-opiate receptor	[^{3}H]D-Ala^2-D-Leu^5-enkephalin binding	≫10	Nickel, 1987
κ-opiate receptor	[^{3}H]Diprenorphine binding, sequential blocking	≫10	Nickel, 1987
μ/δ/κ-opiate receptor	[^{3}H]Diprenorphine binding	≫10	Nickel, 1987
μ/δ/κ-opiate receptor	[^{3}H]Naloxone binding	>10	Nickel, 1987
μ/δ/κ-opiate receptor	[^{3}H]Etorphine binding	>10	Nickel et al., 1985
Benzodiazepine receptor	[^{3}H]Flunitrazepam binding	>10	Nickel et al., 1990b
Basal prostaglandin I2 production	Aorta specimen of the rat	5–50	Darius and Schrör, 1985
Inhibition of arachidonic acid-induced thromboxane production	Platelets, human	>23	Darius and Schrör, 1985
Inwardly rectifying K^+ channel	Patch-clamp, hippocampus	1	Jakob and Krieglstein, 1997

*Kornhuber et al. (publication in preparation). Under therapeutic conditions with serum concentrations of up to about 5 μM, of the effects listed here, only the effect on the inwardly rectifying K^+ channel is relevant

flupirtine is longer in older patients than in young normal subjects; this is accompanied by higher maximum serum concentrations in older patients (Abrams et al., 1988). The same probably applies for preclinical models where pronounced effects are seen with doses of around 1–20 mg/kg in vivo (Block et al., 1994; Carlsson and Jurna, 1987; Schwarz et al., 1994, 1995; Timmann et al., 1995) and 10–20 μM in vitro (Nickel et al., 1990a; Osborne et al., 1994; Perovic et al., 1994; Rupalla et al., 1995). In summary, experimentally determined effects are only clinically relevant if they occur in the low micromolar range.

Pharmacologic effects at therapeutic concentrations

The pain-relieving effect of flupirtine does not appear to be achieved via central opiodergic mechanisms. For example, the analgesic effects of flupirtine are not antagonized by naloxone, and neither are they accompanied by tolerance or physical dependence of the opiate type (Nickel et al., 1985). Moreover, flupirtine does not show any relevant affinity to the opiate receptor system (Nickel, 1987; Nickel et al., 1985) (Table 1) and is also structurally markedly different from morphine. An action via benzodiazepine receptors could also be ruled out (Nickel et al., 1990b). The serotonin receptor antagonist cyproheptadine and the tryptophan hydroxylase inhibitor p-chlorophenylalanine do not inhibit the analgesic properties of flupirtine (Nickel et al., 1985), which also suggests that there is no influence on serotonergic mechanisms. Although no relevant affinities to the α_1- or α_2-adrenoreceptors have been found (Szelenyi et al., 1989), indirect evidence suggests a modulation of pain perception via the descending noradrenergic system (Szelenyi et al., 1989).

The central nervous system effects of flupirtine include analgesia, muscle relaxation and neuroprotection. Antiepileptic and antiparkinsonian properties are also to be found. These properties are only independent of each other at first sight: they are in fact the typical and classical effects of N-methyl-D-aspartate (NMDA) receptor antagonists (Zieglgänsberger and Tölle, 1993; Kornhuber and Weller, 1997). In various indirect studies, flupirtine displayed properties that are consistent with antagonism at the NMDA receptor (Block et al., 1994; Rupalla et al., 1995; Perovic et al., 1994, 1995; Osborne et al., 1994, 1996; Schwarz et al., 1994, 1995), and the recent review articles on flupirtine focus on the NMDA receptor as the main molecular target of flupirtine (Osborne et al., 1998; Schuster et al., 1999). In direct testing, however, it has not been possible to demonstrate a clear interaction with the previously known binding sites of the NMDA receptor (Table 1). In patch-clamp investigations on neuronal cell cultures, flupirtine has no influence on NMDA-induced ion currents (results presented here and Jakob and Krieglstein, 1997). In our own investigations (Kornhuber et al., publication in preparation), flupirtine did not show any relevant affinity to binding sites at the NMDA receptor in human post-mortem brain tissue. Other research groups have reported comparable negative results from binding studies (Osborne et al., 1996, 1998). Recently, it has been suggested that flupirtine interacts with

the redox binding site of the NMDA receptor (Osborne et al., 1998). But this is inconsistent with the negative findings from patch-clamp studies presented here. In summary, it can be stated that flupirtine acts like an NMDA receptor antagonist in functional investigations, although an action at the NMDA receptor could not be found in direct investigations. It is probable that a site of action "up- or downstream" of the NMDA receptor is influenced, leading to a functional NMDA receptor antagonism.

Jakob and Kriegelstein (1997) found an activation of G-protein-regulated inwardly rectifying K^+ channels by flupirtine in therapeutically relevant concentration ranges. According to our current knowledge, this is the only mechanism known to be relevant in a therapeutic concentration range (Table 1). Inwardly rectifying K^+ channels represent a new family of K^+ channels and differ markedly from the classical voltage-dependent K^+ channels. The resting membrane potential is slightly above the K^+ equilibrium potential; a slight outflow of K^+ ions stabilizes the resting membrane potential close to the K^+ equilibrium potential. The overall effect on the cell is a stabilization of the resting membrane potential, e.g. in the case of slight depolarization by excitotoxic stimuli. The G-protein-activated inwardly rectifying K^+ channels (GIRK) are regulated via neurotransmitters, occur in various subtypes and are expressed differently according to the region of the brain involved (Karschin et al., 1994). K^+ channels also play an important role in the transmission of pain stimuli. The analgesic effects of opioids (Ocaña et al., 1990), α_2-adrenergic agonists (Ocaña and Baeyens, 1993), 5-HT_{1A}- agonists (Robles et al., 1996) and other analgetic substances are mediated by receptor-mediated opening of K^+ channels and neuronal hyperpolarization. On the other hand, Substance P inhibits G-protein-dependent K^+ channels (Stanfield et al., 1985) and thus facilitates the transmission of pain stimuli.

Central nervous effects of flupirtine compared to those of experimental K^+ channel openers

Experimental K^+ channel openers like cromakalim display analgesic properties (Ocaña et al., 1996; Robles et al., 1996; Vergoni et al., 1992). Direct evidence of a neuroprotective action of openers of ligand-gated K^+ channels has been found in excitotoxic (Abele and Miller, 1990) and oxidative noxae (Goodman and Mattson, 1996). A study conducted by Lauritzen et al. (1997) shows that cromakalim prevents the glutamate-induced death of hippocampal neurons by counteracting the delayed increase in intracellular Ca^{2+}. An analogy to the effects of flupirtine can be seen here (Zimmer et al., 1998). A stabilization of the resting membrane potential by flupirtine would also be consistent with the initial evidence of antiepileptic properties (Sheridan et al., 1986). Antiepileptic properties have been shown for other K^+ channel openers (Gandolfo et al., 1989; Del Pozo et al., 1990; Popoli et al., 1991). In summary, a comparable central nervous spectrum of action is found for flupirtine and experimental K^+ channel openers. This can be interpreted as additional indirect evidence of an action of flupirtine via an activation of K^+ channels.

Global model and summary

The results obtained so far can be summarized as follows: Therapeutically relevant, analgesic plasma concentrations of flupirtine are in the low micromolar range. In direct investigations, no relevant affinities for α_1, α_2, $5HT_1$, $5HT_2$, dopamine, benzodiazepine, opiate, central muscarinergic, or nicotinic receptors were found. The profile of preclinical and clinical actions (analgesic, muscle-relaxant, neuroprotective, antiepileptic and antiparkinsonian properties) suggests that the action of flupirtine is connected with the NMDA receptor. It has not been possible to convincingly demonstrate a direct action on the NMDA receptor to date. All previous experimental results could be mediated by an indirect influence on the NMDA receptor. Flupirtine acts functionally like an NMDA receptor antagonist. At a therapeutically relevant concentration, flupirtine activates neuronal inwardly rectifying G-protein-regulated K^+ channels. The spectrum of action of the available experimental K^+ channel openers, as far as they have been investigated to date, corresponds to that of flupirtine: These K^+ channel openers also display analgesic, neuroprotective and anticonvulsant properties.

We present a new model to explain the spectrum of action of flupirtine: Flupirtine activates inwardly rectifying K^+ channels and thus stabilizes the resting membrane potential. The Mg^{2+} block of the NMDA receptor remains in force; i.e. the NMDA receptor is indirectly inhibited (Fig. 2). This mechanism provides an explanation for the analgesia, muscle relaxation and neuroprotection. The model on the mechanism of action of flupirtine presented here links neuronal K^+ channels with NMDA receptors via membrane excitability. This provides an understanding of the clinically observed profile of flupirtine's actions, with analgesic, muscle-relaxant and neuroprotective effects. The indirect inhibition of Ca^{2+} inflow into nerve cells, with plastic changes, e.g. in the sense of an increased response ("wind up"), is suppressed by substances like flupirtine. This counteracts the clinically corresponding chronification of pain. From a clinical point of view, flupirtine can be classified as the prototype of a new substance class with analgesic, muscle-relaxant and neuroprotective properties. While flupirtine is clinically used mainly as an analgesic substance, its neuroprotective properties will probably lead to new clinical applications, e.g. in chronic neurodegenerative diseases.

Various questions remain open. Different nerve cells often differ in their intrinsic electrophysiological properties as a result of the differential expression of specific ion channels and their different spatial distribution on the cell surface. In future investigations, it will have to be clarified which subtype of GIRK channels is influenced, in which regions of the brain this takes place, and whether specific receptors, G-proteins or K^+ channels are influenced, and the precise molecular target site will have to be characterized. Cardiac side effects of flupirtine have not been reported to date. This can be interpreted as indirect evidence that flupirtine selectively influences neuronal K^+ channels. This hypothesis must be examined in further investigations. If this hypothesis is confirmed, flupirtine is the prototype of a new substance class, the selective neuronal potassium channel openers (SNEPCO).

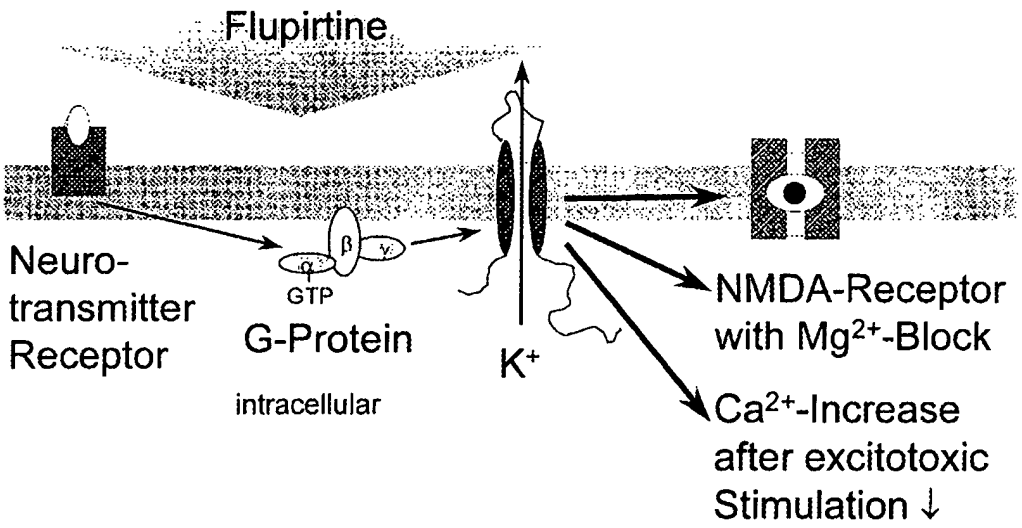

Fig. 2. Flupirtine activates G-protein-coupled inwardly rectifying K^+ channels. The changes under the influence of flupirtine are to be read from left to right. The resting membrane potential is stabilized, an activation of the cell membrane is inhibited. These processes are shown here in different grey tones of the cell membrane: The activated state of the cell membrane (left, dark) is brought into the resting state (right, light) by flupirtine via an activation of inwardly rectifying K^+ channels. An activation of NMDA receptors is prevented, since the Mg^{2+} block of the NMDA receptor is only relieved upon depolarization of the cell membrane. It is conceivable that flupirtine has additional effects that are independent of the NMDA receptor

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1: J Neural Transm. 1999;106(9-10):857-67.

Flupirtine shows functional NMDA receptor antagonism by enhancing Mg2+ block via activation of voltage independent potassium channels. Rapid communication.

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The spectrum of action of flupirtine includes analgesia, muscle relaxation and neuroprotection. N-methyl-D-aspartate (NMDA) receptor antagonism has been discussed as a possible mechanism of action of this compound with little direct evidence. The objective of the present study was to develop a plausible model to explain flupirtine's spectrum of action. A four-stage strategy was selected for this purpose: Firstly, the serum concentration of flupirtine under therapeutic conditions was determined on the basis of the current literature. The second stage involved assessing the known in-vitro effects in light of the therapeutic active concentration. Using whole cell patch clamp recordings from cultured rat superior colliculus neurones interactions between flupirtine and NMDA receptors were assessed. Only very high concentrations of flupirtine antagonized inward currents to NMDA (200 microM) at -70 mV with an IC50 against steady-state responses of 182.1+/-12.1 microM. The effects of flupirtine were voltage-independent and not associated with receptor desensitization making actions within the NMDA receptor channel or at the glycine modulatory site unlikely. NMDA receptor antagonism probably has little relevance for the clinical efficacy of flupirtine as the concentrations needed were far higher than those achieved in clinical practice. However, the activation of a G-protein-regulated inwardly rectifying K+ channel was identified as an interesting molecular target site of flupirtine. In the next stage, the central nervous spectrum of action of experimental K+ channel openers (PCO) was considered. As far as they have been studied, experimental K+ channel openers display a spectrum of action comparable to that of flupirtine. In the final stage, a global model was developed in which flupirtine stabilizes the resting membrane potential by activating inwardly rectifying K+ channels, thus indirectly inhibiting the activation of NMDA receptors. The model presented here reconciles the known functional NMDA receptor antagonism of flupirtine with the activation of K+ channels that occurs at therapeutic concentrations, thus providing an understanding of flupirtine's spectrum of

action. This makes flupirtine the prototype of a clinically applicable substance group with analgesic, muscle-relaxant and neuroprotective properties.

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